TRANSCRIPTIONAL REGULATION OF THE MINERALOCORTICOID RECEPTOR IN RESPONSE TO CELLULAR INJURY

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DECLARATION

I hereby declare that this thesis was composed by myself and the data presented represent my own work, with the exceptions listed below:

The hypothermic anoxic neonatal rats were generated by Dr. Justyna Rogalska in Department of Animal Physiology, Institute of General and Molecular Biology, Copernicus University, Poland.

The MRα promoter construct was generated by Dr. Maija Castren in University of Kupio, Finland.

The plasmid template for generating riboprobe targeting total MR in neonatal rat *in vivo* was kindly provided by Dr. Joyce Yau, Cardiovascular Science, QMRI, University of Edinburgh, UK.

The plasmid pCH110 encoding β-galactosidase and undifferentiated PC12 cells were generously provided by Prof. Karen Chapman. Cardiovascular Science, QMRI, University of Edinburgh, UK.

I declare that this work has not been submitted for any other degree.

Peng Kang, Edinburgh 2009

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LIST OF PUBLICATIONS AND PRESENTATION

Publications

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Rogalska, J., Kang, P., Wotherspoon,W., Macleod, MR and Lai, M (2008). Effect of hyperthermia and anoxia on glucocorticoid and mineralocorticoid receptor expression in neonatal rat hippocampus. Neurosci Lett. 2009 Jan 30;450(2):196-200

Abstracts

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LIST OF ABBREVIATIONS

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
5-HT	5-hydroytryptamine
ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphotase
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
CA1	*Cornu Ammonis*
CaMK	Calcium/calmodulin kinase
Caspases	*c*ysteine-*asp*artic acid prote*ases*
cDNA	complementary DNA
CHIF	Channel-inducing factor
CIAP	Calf intestinal alkaline phosphatase
c.p.m	Counts per minute
CREB	cAMP response element binding protein
CRH	Corticotropin-releasing hormone
DAB	Diaminobenzidine
Dex	Dexamethasone
DEPC	Diethyl pyrocarbonate

DG	Dentate gyrus
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EC	Effective concentration
EDTA	Ethylene diamine tetra-acetic acid
Elk-1	Ets-like transcription factor-1
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
ETS	E26-AMV virus oncogene cellular homolog
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FKBP	FK506 binding protein
GC	Glucocorticoid
GFAP	Glial-fibrillary acidic protein
GR	Glucocorticoid receptor
GRE	Glucocorticoid-response element
H_2O_2	Hydrogen peroxide
HBSS	Hank's buffered saline solution
HIF	Hypoxia-inducible factor
hMR	Human mineralocorticoid receptor
HPA	Hypothalamus-pituitary-adrenal gland
HRE	Hormone response element
HS	Horse serum
HSP	Heat shock protein
IAPs	Inhibitor of apoptosis proteins
IL	Interleukin

JNK	c-Jun N-terminal kinase
kb	Kilobase
LB	Luria-Bertoni
LDH	Lactate dehydrogenase
LTP	Long-term potentiation
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinases
ml	Milliliter
MPT	Mitochondrial permeability transition
MR	Mineralocorticoid receptor
MRE	Mineralocorticoid-response element
MRI	Magnetic resonance imaging
mRNA	messenger Ribonucleic acid
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NGF	Nerve growth factor
NHS	National Health Service
nm	Nanometre
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
nt	Nucleotides
NT-3	Neurotrophin-3
NT-4/5	Neurotrohphin-4/5
$O_2^{\cdot-}$	Superoxide anion radicals
OGD	Oxygen-glucose deprivation
OH^-	Hydroxyl radicals
ONPG	Nitrophenyl-β-galactoside

PBS	Phosphate buffered saline
PC12	Pheochromocytoma (designated as 12)
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinases
PKA	Protein kinase A
PKC	Protein kinase C
PVN	Paraventricular nucleus
RAAS	Renin-angiotensin-aldosterone system
rMR	Rat mineralocorticoid receptor
RNA	Ribonucleic acid
RPA	Ribonuclease protection assay
r.p.m	Round per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
rt-PA	recombinant tissue plasminogen activator
SAPK	Stress-activated protein kinase
S.E.M	Standard error of the mean
SGK1	Serum glucocorticoid-regulated kinase 1
STAIR	Stroke Therapy Academic Industry Round Table
STS	Staurosporine
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
tRNA	Transfer Ribonucleic acid
VEGF	Vascular endothelial growth factor
XIAP	X chromosome-linked inhibitor of apoptosis protein

ABSTRACT

Neurons are particularly vulnerable to hypoxic-ischaemic injury and this injury which occurs in human brain can lead to death and long-term disability in survivors. At the cellular level, such events can result in the activation of a number of pathways such as PI3K/Akt and ERK that can protect neurons from subsequent insults; some of these effects require changes in gene expression. One possible neuroprotective strategy against the neuronal damage associated with hypoxia-ischaemia is to identify and augment these protective changes in gene expression.

Glucocorticoid hormones are potent regulators of neuronal viability with actions via both glucocorticoid (GR) and mineralocorticoid receptors (MR). While GR signalling is known to endanger neurons by making them more vulnerable to the toxic effects of various insults, predominant MR activation is thought to promote neuronal survival. Recently MR expression has been shown to be rapidly increased in response to different types of injury both *in vitro* and *in vivo* and this is associated with enhanced survival, suggesting a novel endogenous survival mechanism. A causal link between increased MR expression and neuroprotection has since been demonstrated following transgenic MR overexpression both *in vitro* and *in vivo*, suggesting that increasing MR signalling may have important therapeutic uses in patients at high risk of hypoxic-ischaemic insults.

This study has aimed to increase our knowledge of the mechanisms underlying the transcriptional regulation of MR. The rat MR gene gives rise, through alternative splicing, to three distinct MR mRNA transcripts (MRα, β and γ) which differ in their 5'-untranslated regions; each 5'-flanking region operates as a functional promoter. While MR mRNA expression is known to be modulated through autoregulation and some compounds such as serotonin and progesterone, the

mechanism(s) by which injury-induced MR occurs and whether there is differential regulation of the transcripts is not known.

Transcription of each MR variant was measured in various *in vitro* models of cell injury at both the promoter and mRNA level. The results at promoter level suggest that MRα and MRβ variants are responsive to some but not all types of cell injury while MRγ appears to be unaffected under any condition in neuronal-like cells. Measurement of mRNA abundance in primary cortical culture subjected to the same cellular stressors identifies only MRβ is the primary splice variant.

The abundance of each brain MR variant was also measured in an *in vivo* injury model of hypothermic anoxic injury in neonatal rats where damage occurs predominantly in the hippocampus – the region with the highest expression of MR. Levels of total MR mRNA were significantly increased in all regions of the hippocampus under hypothermic conditions (except CA2) and further increased in the presence of anoxia, which is contributed by MRβ solely. These observations corroborate the *in vitro* data in demonstrating a key role for the MRβ variant in response to ischaemic injury and support the hypothesis that some of neuroprotective effects of hypothermia are mediated via MR.

Overall, the data suggests that increased MR expression is not a generic response to injury and that there is indeed differential regulation of the transcripts depending on the type of injury, which exclusively via the expression of MRβ. This provides a greater understanding of the regulation of MR in the context of brain cell injury and should contribute to the future clinical exploitation of the protective effects of MR in patients at high risk of cerebral ischaemia.

Chapter 1

INTRODUCTION

Men ought to know that from nothing else but the brain come joys, delights, laughter and sports, and sorrows, griefs, despondency, and lamentations. And by this, in an especial manner, we acquire wisdom and knowledge, and see and hear and know what are foul and what are fair, what are bad and what are good, what are sweet and what are unsavory.....

Hippocrates, *On the Sacred Disease* (4th century B.C.)

1.1 The structure and function of the brain

Perhaps the most sophisticated organ in humans is the brain – the control centre of the body – which regulates the flow of information to receive and act with speed in a coordinated manner in response to internal or external stimuli. This includes involuntary reactions such as breathing, digestion and heart beat, and voluntary actions, such as talking and walking. In particular, the brain is the seat for intelligence that distinguishes human beings from other mammalian animals.

The human brain is composed of four principle sections: the cerebrum, diencephalon, cerebellum and brain stem. *The cerebrum* is the largest and most well-developed section in the mammalian brain, particularly in humans and is involved in directing movement, sensory processing, and other more complex functions such as perceptual awareness, emotion, language, learning and memory. *The diencephalon* comprises the thalamus and hypothalamus: the thalamus serves to process all sensory input to the cortex while the hypothalamus exerts control over the pituitary gland and subsequently the endocrine system. Together the cerebrum and diencephalon are known as the forebrain. *The cerebellum* is the hindbrain structure, which mainly participates in the integration of sensory perception, coordination and motor control. *The brain stem* is the conduit for the major tracts that pass the information on the way to or from the brain. It also plays a vital role in basic attention and consciousness and is critical for cardiovascular and respiratory control (Siegel *et al.* 1998).

1.1.1 Neurons

The brain is largely composed of two broad classes of cell, neurons and glia. Although neurons are outnumbered by glia by 10-fold, they are the primary

responsive cells. Like other cells in the body, neurons have common cellular organelles; for example, nucleus, endoplasmic reticulum, Golgi complexes and mitochondria, to maintain the normal cellular activities (Siegel *et al.* 1998). These are contained in the central part of the neuron called the soma. However, neurons are highly specialized in that they contain two unique features: axons and dendrites. Dendrites are cellular extensions of the soma and the sites of input from neighbouring neurons. Axons are usually a single projection from the soma which can project up to a thousand times the length of the soma and are responsible for carrying nerve signals away from the soma to another neuron (Bear *et al.* 2007).

Neurons process information by conducting electrical impulses through the movement of electrical charge in the form of chemical ions (Na^+, K^+, Ca^{2+} and Cl^-) across the cell membrane (Siegel *et al.* 1998). Under resting conditions, neurons maintain high concentrations of extracellular Na^+ and Ca^{2+} and intracellular K^+ through selective ATP-dependent ion channels in the membrane: such as the Na^+-K^+ pump and Ca^{2+} pump (Armstrong 1981; Catterall 1991).

Upon chemical or mechanical stimuli, Na^+ channels are opened causing Na^+ influx into the neuron to depolarise the neuron (Armstrong 1981). This has the effect of opening voltage-gated K^+ channels to allow K^+ ions to leave the neuron and depolarise the membrane. This cycle of depolarisation/repolarisation is propagated along the length of the axon to the terminal where the electrical impulse is converted to a chemical signal through the release of a specific neurotransmitter into the synaptic cleft to communicate with the neighbouring neuron (Siegel *et al.* 1998).

1.1.2 Glia

Glial cells are the non-neuron cells of the brain and do not conduct electrical impulses. However their presence is essential for the proper functioning of the neurons and they can be sub-divided into macroglia (astrocytes and oligodendrocytes) and microglia. Overall, glial cells are present in much greater abundance in the brain and their major function is to provide metabolic support in the form of nutrient and oxygen supply, maintain homeostasis by regulating the chemical environment, provide structural support and an immune defence system (Ransom *et al.* 2003; Volterra *et al.* 2005; Haydon *et al.* 2006).

1.1.3 Metabolic demands of the brain

As a result of a high state of metabolic activity, neurons have a high demand for energy. Unlike most other organs which are considerably flexible to different energy substrates, the brain exclusively utilizes glucose and oxygen to meet its energy demands in the form of ATP production via the mitochondrial respiratory chain. Approximately 4×10^{21} molecules (7mmol) of ATP per minute are estimated to be generated in the entire human brain (Siegel *et al.* 1998). However this is consumed almost as quickly as it is generated because the brain is unable to store ATP. Under normal conditions, a fine balance is struck between the rate of ATP produced and utilized.

The majority of the ATP is used to power the ion pumps which regulate the ion flux across the neuronal membrane to form the action potential and therefore generate electrical conductance. For example, as much as 70% of the ATP is required by the Na^+/K^+ pump itself whereas the energy expenditure for protein and lipid synthesis, turnover of neurotransmitters, aminophospholipid translocation

and Ca^{2+} transport accounts for only a few percent (Siegel *et al.* 1998). Since the brain relies only on oxygen and glucose as energy substrates, a lack in their supply such as a reduction of blood flow into the brain can have serious consequences (Hansen *et al.* 1988).

1.2 Cerebral ischaemia

1.2.1 Definition

Cerebral ischaemia is a condition in which the whole or some part of the brain loses the normal function due to lack of glucose and oxygen as a result of reduced blood flow to the brain. It can be a result of various diseases such as stroke, brain trauma, epilepsy and cardiac arrest and is the most common cause of neurological death or disability (Mackay *et al.* 2004). For example, ischaemic stroke ranks as the third leading cause of death throughout the world, only behind coronary heart disease and cancer (Young *et al.* 1995; Mackay *et al.* 2004).

1.2.2 Classification

Cerebral ischaemia can be broadly grouped into three categories: global, focal and diffused hypoxia (Figure 1-1). In scientific research, this classification is also referred to when generating the animal models *in vivo*.

Global cerebral ischaemia involves the stoppage of blood supply to most or all of the brain. It is commonly caused by cardiac arrest, ventricular fibrillation and coronary artery bypass surgery and results in the neuronal injury to selectively vulnerable regions of the brain. If global ischemia continued indefinitely, then all

neurons would die. Several rodent models of global cerebral ischaemia exist, produced mainly by temporary vessel occlusion of vertebral arteries and/or common carotids (Traystman 2003). The occlusion time is dependent on the species but in general 5–20 minutes is most commonly used and ischaemia is subsequently followed by a period of reperfusion. The levels of ATP fall quickly during global cerebral ischaemia. For example a 2-minute occlusion results in more than an 85% reduction in ATP in both the gerbil and rat (Ljunggren *et al.* 1974; Ekholm *et al.* 1992) whereas the energy levels are generally restored quickly once reperfusion occurs (Pulsinelli *et al.* 1983).

The key feature of global cerebral ischaemia is a substantial delay between the short insult and neuronal death, which generally happens between 12 hours and several days after reperfusion (Pulsinelli *et al.* 1979; Kirino 1982; Pulsinelli *et al.* 1982). This delayed neuronal death depends on the duration and severity of the insult and the cell population in the vulnerable regions like pyramidal cells in hippocampal CA1 subfield (Kirino 1982; Crain *et al.* 1988).

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Focal cerebral ischaemia is defined as the blockage of blood in an artery supplying a specific region of the brain, which represents a typical clinical ischaemic stroke. It is generally caused by an embolus from a plaque in a large artery, or a thrombus in the heart, or a platelet plug formed directly in the affected artery. In focal ischaemia, the area of the lesion is typically smaller than the entire distribution of the occluded artery, due to the blood supply from the collateral circulation at the borders of the region.

Focal cerebral ischaemia in nonhuman primate and rodent models is primarily generated by occlusion of one major cerebral artery, usually the middle cerebral artery (Symon 1975; Strong *et al.* 1983; Ginsberg *et al.* 1989; Traystman 2003).

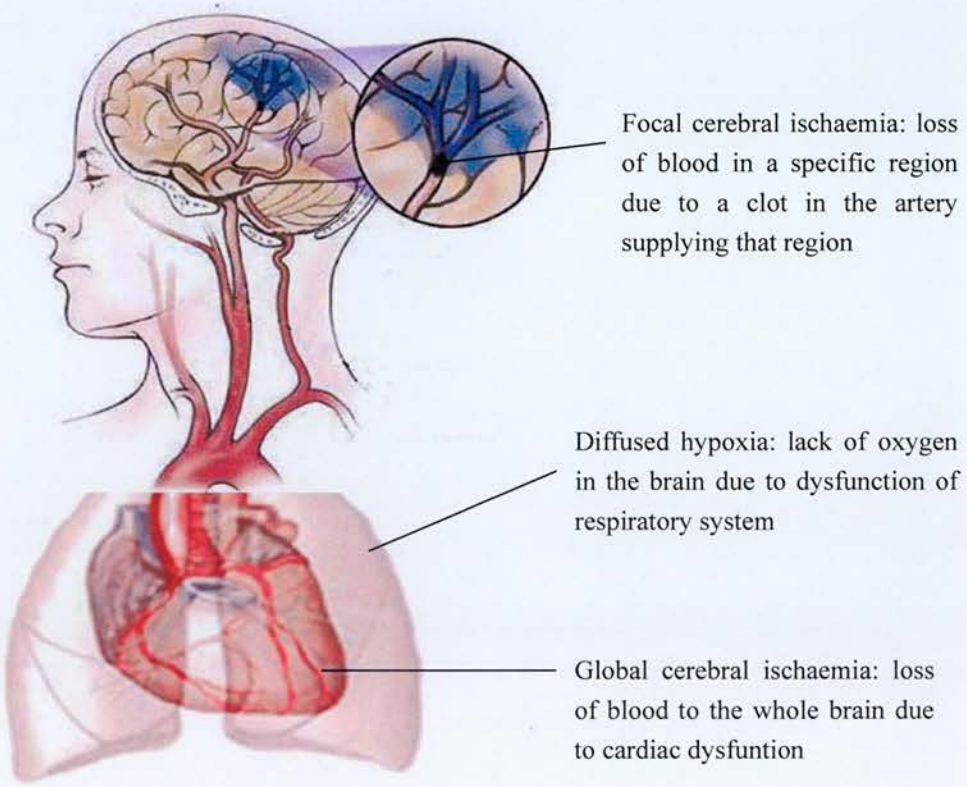


Fig 1-1 Classification of cerebral ischaemia. Three types of cerebral ischaemia are broadly present: global, focal cerebral ischaemia and diffused hypoxia.

Therefore the damage is caused selectively to the striatum and cortex which are the main regions fed by the middle cerebral artery. Focal cerebral ischaemia models can either be temporary or permanent and both types have been used extensively because the subsequent pathology is highly relevant to human ischaemic stroke (Traystman 2003). The unique feature of this type of ischaemia is the formation of an 'ischaemic core', where affected neurons die within seconds to minutes (Siesjo 1992; Lipton 1999). However, neurons around the rim of the core, termed as the "penumbra", are damaged and can take up to a few days to die therefore potentially penumbral neurons can be rescued (Lipton 1999). The extent of the ischaemic core and penumbra is determined by the duration and the degree of vascular occlusion (Greenfield *et al.* 2002) and these parameters can be easily manipulated in the animal models.

Focal cerebral ischaemia causes a rapid and extensive depletion of ATP levels down to 25% or less in the core between 5-minute to 4-hour occlusion (Welsh *et al.* 1991; Folbergrova *et al.* 1992; Folbergrova *et al.* 1995). However, levels of ATP in the penumbra drop less rapidly, generally down to 50–70% after 1-hour ischaemia (Welsh *et al.* 1991; Folbergrova *et al.* 1995; Sun *et al.* 1995). In temporary focal ischaemic models where reperfusion occurs, ATP levels are rarely back to normal pre-ischaemic values (Ikonomidou *et al.* 1989; Kleiman *et al.* 1990; Lipton 1999).

Ccerebral hypoxia refers to oxygen deprivation to the brain. Mild or moderate hypoxia is known as diffused cerebral hypoxia which can be caused by airway obstruction, pulmonary oedema, altitude sickness or severe anaemia whereas total deprivation of oxygen is more commonly referred to cerebral anoxia. Hypoxia has been most widely used in neonatal animals that reliably survive for days after exposure to such insult, to mimick major forms of neonatal metabolic brain damage (Vannucci 1990) and for the majority, the oxygen level is generally

lowered to 8% or even less (Rice *et al.* 1981; Roohey *et al.* 1997). Hypoxia normally induces cerebral dysfunction rather than irreversible damage. However, similar to global and focal cerebral ischaemia, neuronal fate is determined by the period of exposure and the severity of the insult for instance in young rats. 60 minutes of hypoxia at 8% oxygen level has been shown to reduce ATP levels by 30–50% (Williams *et al.* 1994). Much like global cerebral ischaemia, 15–30 minutes of hypoxia at 3% caused early ischaemic cell change and delayed neuronal loss in CA1 to CA3, striatum and layer 5 of cortex in adults (Salford *et al.* 1973).

1.2.3 Molecular pathophysiology of cerebral ischaemia

The critical step in cerebral ischaemia is the reduction of ATP levels which leads to a cascade of cellular events that eventually cause neuronal dysfunction and if severe, neuronal death. Using different *in vitro* and *in vivo* models, the pathophysiology of ischaemic neuronal death has been largely identified at cellular and molecular levels.

1.2.3.1 Ion disturbance

Loss of ATP primarily affects the function of the ion pumps; for example, Na^+-K^+ ATPase. Failure of Na^+-K^+ ATPase allows the high concentrations of intracellular Na^+, a state opposite to the normal physiological condition, inducing a long-lasting membrane potential loss and neuron depolarisation.

1.2.3.2 Ca^{2+} overload

Physiological extracellular concentrations of Ca^{2+} are several thousand times higher than intracellular concentrations and Ca^{2+} enters the cells mainly via two types of channels: voltage-controlled and receptor-operated Ca^{2+} channels (Miller 1987). The export of Ca^{2+} depends on the ATP directly (ATP-dependent Ca^{2+} channel) or indirectly (Na^{+}-Ca^{2+} exchanger) (Baudry *et al.* 1981).

During ischaemia, intracellular concentration of Ca^{2+} is significantly increased, which is contributed by several major factors:

(i) Accumulated Na^{+} inside the cells due to inhibition of Na^{+}-K^{+} pump significantly exchanges more Ca^{2+} from extracellular environment (Carini *et al.* 1994).

(ii) The depolarisation of the neuronal cell membrane triggers the opening of voltage-gated Ca^{2+} channels that allows more Ca^{2+} influx.

(iii) The combination of ATP breakdown and depolarisation greatly impairs the reuptake process for neurotransmitter glutamate (Wahl *et al.* 1994). This leaves excessive glutamate to long-lasting activation of the ionotropic NMDA and AMPA/kainite receptors, resulting in a further energy crisis and longer membrane depolarisation. As the receptor-gated Ca^{2+} channel, the activation of NMDA receptor also induces more Ca^{2+} influx into the cells (Budd 1998).

(iv) The ATP-dependent Ca^{2+} exporting systems are inactive due to energy failure, leaving a high concentration of intracellular Ca^{2+} in the cytoplasm and other cellular organs, which initiates a number of signalling pathways leading to cell death.

The consequences of Ca^{2+} overload are enormous because calcium is a universal second messenger and activates a series deteriorating effects in cells.

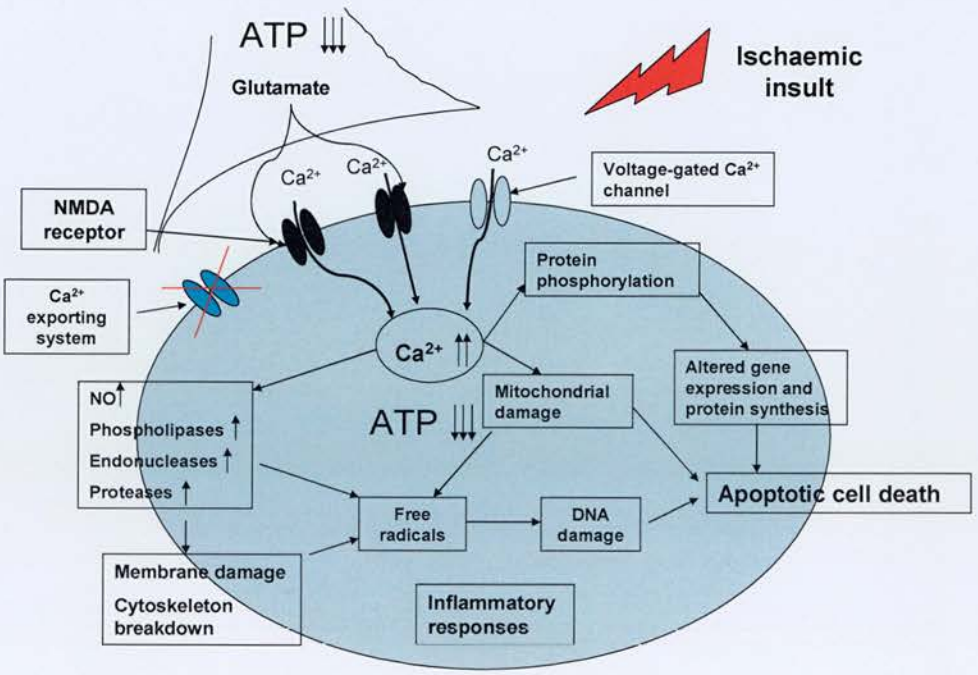


Fig 1-2 Schematic illustration of pathophysiolgoical progress in ischaemia-induced cell death.

Breakdown of cell membrane and cytoskeleton

Phospholipases

Phospholipases are a group of enzymes that catalyze the hydrolysis of membrane phospholipids. The activation of phospholipase A_2 is mediated by Ca^{2+} and involved in fatty acid synthesis. In global cerebral ischaemia, the expression of phospholipase A_2 was enhanced and followed by an increased level of free fatty acid (Lauritzen *et al.* 1994; Chan 1996; Pilitsis *et al.* 2002). The increased fatty acid is believed to be substantially neurotoxic and detrimental to the membrane integrity (Yang *et al.* 2007).

Endonucleases

Brain ischaemia can cause both non-specific and specific DNA cleavage during the course of cell death. Although the mechanism of non-specific DNA degradation it is not clear, evidence has shown that the endonuclease-mediated specific cleavage was mediated by Ca^{2+} overload (Tominaga *et al.* 1992).

Proteases

Calpains are cycteine proteases that widely expressed in cytoskeletal systems and require Ca^{2+} for activation (Mellgren 1987). Calpain I was considered as the most important protease to promote ischaemic-induced cell death, according to previous studies on its location and role in episode of ischaemic cell death (Kirino *et al.* 1984; Smith *et al.* 1984; Hamakubo *et al.* 1986; Perlmutter *et al.* 1990).

Nitric oxide

Nitric oxide (NO) is a prominent vascular and neuronal messenger molecule formed directly from the guanidine nitrogen of L-arginine by nitric oxide synthase (NOS) (Garthwaite 1991). In the brain, NOS has been identified as a monomer of 150kDa inactive protein at basal Ca^{2+} concentration, while being activated through increased Ca^{2+} level (Bredt *et al.* 1990). The pathogenic importance of NO is

reflected in a number of observations that pharmacological inhibitors of inducible NOS reduce ischaemic damage to the brain because the activation of NOS produces more toxic NO. Low doses of NOS inhibitor nitroarginine reduce infarct volume following focal ischaemia in rats (Buisson *et al.* 1992) and mice (Nowicki *et al.* 1991). Transgenic mice lacking expression of neuronal NOS showed a reduced infarct size following ischaemia (Samdani *et al.* 1997).

Mitochondrial damage

Mitochondrial damage plays an important role in cell death during cerebral ischaemia (Kluck *et al.* 1997; Kroemer *et al.* 1998). A large body of evidence suggests mitochondrial Ca^{2+} accumulation is a major mechanism of mitochondrial damage and cytotoxicity (Ankarcrona *et al.* 1995; Schinder *et al.* 1996). A decrease in the mitochondrial membrane potential and ATP depletion due to cytosolic Ca^{2+} accumulation is initiated as the first event (Dubinsky *et al.* 1998). Furthermore, the inhibition of mitochondrial Na^{+}-Ca^{2+} exchanger, which normally pumps the excessive Ca^{2+} from the mitochondria into cytoplasm, enhances this cellular pathological process (Nicholls *et al.* 1998). Subsequently, these pathological changes lead to the breach of mitochondrial permeability.

The mitochondrial permeability transition (MPT) is characterized by a sudden increase in the permeability of the mitochondrial inner membrane to small ions and molecules (Haworth *et al.* 1979; Gunter *et al.* 1994). It was originally suggested by Nicholls that phospholipase A_2 activation broke down the lipid backbone of the inner membrane, giving rise to a non-specific increase in mitochondrial membrane permeability (Nicholls 1985). However, a different mechanism was presented shortly after, that a prolonged opening of a proteinaceous pore due to the enhanced affinity to the internal site by mitochondrial Ca^{2+} is more relevant for this permeability transition (Bernardi *et al.*

1992; Connern *et al.* 1992; Gunter *et al.* 1994). Loss of membrane potential can also increase the MPT pore opening (Petronilli *et al.* 1994). The assembly of the MPT pore quickly releases Ca^{2+} and other molecules from intra-mitochondrial compartments and subsequently the total collapse of electrical potential difference, allowing the regulated entry of H^+ across the inner mitochondrial membrane that is essential for respiratory chain reaction. Therefore, ATP production is ceased and osmotic swelling happens in the mitochondria. In addition, mitochondria damage can produce large quantities of reactive oxygen radicals (Zamzami *et al.* 1995).

Protein phosphorylation and gene activation

Ca^{2+} influx is responsible for the activation of a number of protein kinases such as protein kinase C (PKC) (Domanska-Janik *et al.* 1992; Fukunaga *et al.* 1992), calcium/calmodulin kinase II (CaMK II) (Aronowski *et al.* 1992; Churn *et al.* 1992), cAMP-dependent protein kinase (PKA) (Hu *et al.* 1994) etc. These protein kinases lead to the activation of a variety of signalling pathways such as CREB (Corcoran *et al.* 2001) and MAPK (Hu *et al.* 1994) to mediate the downstream gene expression that can determine the cell fate.

1.2.3.3 Free radicals formation

Free oxygen radicals are molecules that possess an outer electron orbital with a solitary unpaired electron. They are produced in small quantities during aerobic metabolism in mitochondria. Superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are formed during the mitochondrial electron transport chain and are extremely toxic to the cells (Siesjo *et al.* 1989). During ischaemia, insufficient O_2 is available to accept the electron from the respiratory chain, resulting in the excessive production of $O_2^{\cdot-}$. More hydroxyl radicals (OH^-) may be generated from

H_2O_2 by the release of iron from ferritin stores and damaged neurons (Halliwell 1992). Except for hydroxyl radicals, nitric oxide is also another form of oxidative free radicals.

The major consequence of oxidative free radicals is to damage the cellular macromolecules. For example, the addition of the free 'radicals' electron to a fatty acid causes fragmentation of the lipid or alteration of its chemical structure, known as lipid peroxidation, which acts as one detrimental factor to breakdown the cell membrane (Halliwell 1992). Besides, the other means by which free radicals damage the proteins are cross-linking between amino acids, carbonyl formation and protein denaturation. DNA bases can also be modified by oxidation, leading to non-specific cleavage and mispairing of purine and pyrimidine during DNA replication (Cao *et al.* 1988; Halliwell 1992).

In addition to inducing injury directly to the cells, oxidative free radicals may indirectly affect several pathological changes; for example, enhancement of NMDA receptor-mediated excitotoxicity, contribution to initiation and early signals of apoptotic neuronal death and interaction with other multiple injuries like inflammation (Siesjo *et al.* 1989).

1.2.3.4 Inflammatory responses

Inflammatory responses by ischaemia are complex and involve a lot of inflammatory factors. Evidence showed that a number of inflammatory mediators are induced by ischaemia in astrocytes, microglia, leukocytes and endothelial cells as the instant initiator (Rothwell 1997), including cytokines, chemokines and adhesion molecules (Feuerstein *et al.* 1998). Cytokines such as TNF-α and IL-1β are responsible for the accumulation of inflammatory cells in the injured brain and

affect the survival of damaged neurons. For example, the recruitment of leukocytes by TNF-α may promote the infarction size by their toxic by-products and phagocytic action (del Zoppo *et al.* 1991; Kochanek *et al.* 1992).

1.2.3.5 Apoptosis

Neurons that suffer irreversible damage induced by ischaemia will eventually undergo apoptosis, a process of programmed cell death (Goto *et al.* 1990), which minimizes the release of genetic materials and other pro-inflammatory intracellular constituents (Johnson *et al.* 1993). Apoptosis is characterized by apparent morphological changes, including chromosome condensation and aggregation to the nuclear margin, cell body shrinkage, fragmentation of the nucleus and cytoplasm into membrane-bound vehicles (Kerr *et al.* 1972; Wyllie *et al.* 1980). It is triggered by a number of cellular cascades, such as free radicals, mitochondrial damage, phosphorylation of some kinases and intracellular Ca^{2+}. Release of cytochrome c from damaged mitochondria is an important step on the progress of ischaemia-induced apoptosis (Fujimura *et al.* 1998; Sugawara *et al.* 1999). Upon release, cytochrome c is translocated from mitochondria to cytoplasmic compartment and activates a number of specific downstream caspases to trigger apoptosis.

1.2.3.6 Necrosis

It is worth mentioning that neurons in ischemic brain also undergo another form of cell death termed as necrosis, characterized by loss of membrane integrity, disruption of intracellular organelles, cellular swelling and lysis (Lipton 1999; Fujita *et al.* 2003). Unlike apoptosis, the molecular mechanisms of necrosis still remain to be fully elucidated, while some evidence has emerged that it is closely

related to the energy failure or drastic loss of cellular ATP levels (Eguchi *et al.* 1997; Leist *et al.* 1997). Necrotic cell death happens predominantly in the ischemic core of focal cerebral ischemia, in comparison to apoptosis which is mainly responsible for delayed cell death in focal ischemic penumbra and global hypoxic/ischemia (Lipton 1999). In addition, necrosis occurs rather quickly within few seconds to minutes after the onset of ischemia and can not be reversed by any means insofar (Ueda *et al.* 2004), therefore it is not discussed in this thesis.

1.2.4 Neuronal survival in cerebral ischaemia

At the same time that cerebral ischaemia triggers the cascade of events that leads to neuronal death, neurons also mount their own endogenous survival mechanisms in an attempt to restore homeostasis. Therefore the balance between activation of death and survival pathways determines the fate of the neuron. For the most part, activation of endogenous protective mechanisms also requires changes in gene expression.

1.2.4.1 Endogenous survival factors

Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF) contains a family of growth factors involved in angiogenesis, wound healing and embryonic development. In the brain, bFGF is widely expressed in both neurons and glial cells. It has been shown that expression of bFGF was markedly increased in the hippocampi of rats following transient global ischaemia (Takami *et al.* 1992; Endoh *et al.* 1994). In *in vitro* rat hippocampus culture, bFGF pre-treatment promoted long-term survival and significantly enhanced the resistance of neurons to glutamate and NMDA toxicity

(Morrison *et al.* 1986; Mattson *et al.* 1993), glucose deprivation (Cheng *et al.* 1991) and hypoxia (Mattson *et al.* 1993). This neuroprotective effect of bFGF was also found in *in vivo* animal models of ischaemia (Nakata *et al.* 1993; Koketsu *et al.* 1994).

Neurotrophins

Neurotrophins (or neurotrophic factors) belong to the family of growth factors. Due to their specific functions in the brain, in this thesis neurotrophins are listed as a separate part. In general, neurotrophins include four structure-related proteins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Each of these neurotrophins is expressed in the brain in distinct region and cellular patterns. For example, BDNF is produced in all neuronal cells, while NGF is only expressed at high level in hippocampal neurons (Hennigan *et al.* 2007). It is accepted that neurotrophins mediate the neuronal development and are in trophic support of adult neurons under normal circumstances. While in brain injuries like ischaemia, the expression of neurotrophins in particular BDNF and NGF can be increased to significant levels (Lindvall *et al.* 1992). The induction of neurotrophins was followed by the attenuated neuronal loss in various cerebral ischaemia models. For example, the intraventricular administration of NGF protected hippocampal neurons against ischaemic injury in rats (Shigeno *et al.* 1991). NT-3 and BDNF rescued the hippocampal, septal and cortical neurons against glucose-deprivation (Cheng *et al.* 1994). Therefore, the expression of neurotrophins is essential for rescuing neurons at risk of death.

Hypoxia-inducible factor-1 (HIF-1)

HIF-1 is a transcription factor playing a central role in cellular regulation of a

broad variety of hypoxia/ischaemia-induced genes (Semenza 1998). In cell nucleus, HIF-1 binds to specific DNA sequence in enhancers and promoters of the target genes such as erythropoietin, some glucose transporters, several glycolytic enzymes and vascular endothelial growth factor (VEGF) (Ebert *et al.* 1995; Semenza 1998; Wood *et al.* 1998). Accumulation of HIF-1 was thought to contribute to the neuroprotection of the neonatal brain against hypoxia/ischaemia by adaptation to this insult, such as regulating VEGF (Ikeda *et al.* 1995; Forsythe *et al.* 1996). glucose transport (Gidday *et al.* 1994) and glycolysis (Wood *et al.* 1998).

Endogenous inhibitors of apoptosis

Inhibitors of apoptosis proteins (IAPs) are endogenous anti-apoptotic proteins which potently inhibit prominent caspases (caspase-3, -7 and -9) in apoptotic pathways mediated by mitochondria. X chromosome-linked inhibitor of apoptosis protein (XIAP) is a particularly interesting target and has been investigated well in previous studies. XIAP was found significantly increased in the brain after hypoxia-ischaemia by cardiac arrest (Katz *et al.* 2001). Another study showed the protein level of XIAP was enhanced in the hippocampal homogenate in rats 24 hours following cerebral ischaemia and suggested that as the checkpoint in the progress of apoptosis (Siegelin *et al.* 2005). Viral-mediated over-expression of XIAP in the rat hippocampus prevented CA1 neurons from degeneration after transient forebrain ischaemia (Xu *et al.* 1999). Transgenic mice overexpressing human XIAP in neurons displayed a smaller lesion size, less reduction in protein synthesis and less active pro-apoptotic caspase-3 after cerebral ischaemia (Trapp *et al.* 2003). Therefore, XIAP plays a vital role in mediating apoptosis progress and it itself is a neuroprotective factor.

Heat shock protein (HSP)

HSPs are highly conserved molecular chaperones in bacteria, plant and animals which bind intracellular proteins and prevent their inappropriate folding (Nowak *et al.* 1994). The 70kD member of HSP family, HSP70, has received most attention. Cerebral ischaemia markedly increased the expression of HSP70 in the neurons (Nowak *et al.* 1994). Magnetic resonance imaging (MRI) studies suggested that overexpression of HSP70 reduced overall lesion size and may limit the tissue damage within the lesion in permanent cerebral ischaemia (van der Weerd *et al.* 2005). Conversely, mice lacking the HSP70 gene have increased infarction and apoptotic cell death in transient focal ischaemia (Lee *et al.* 2004). The mechanism of HSP70-induced neuroprotection was widely believed to be its chaperone activity at first, while new evidence has emerged that HSP70 can also inhibit the release of cytochrome c from damaged mitochondria (Tsuchiya *et al.* 2003), sequester the apoptosis-inducing factor (Matsumori *et al.* 2005) and modulate the JNK-mediated apoptotic signalling (Meriin *et al.* 1999; Park *et al.* 2001).

Anti-apoptotic protein in Bcl-2 family

Bcl-2 family contains a number of intracellular proteins regulating apoptosis and over 30 members of this family have been identified in higher eukaryotes to date (Borner 2003). Despite of the controversial points on the mechanisms, many researchers believe that Bcl-2 family proteins can directly regulate the MPT pore through which cytochrome c is released (Shi 2001). Anti-apoptotic proteins like Bcl-2 and Bcl-xL can maintain the mitochondrial membrane potential by MPT pore and block the cytochrome c release while pro-apoptotic proteins such as Bax, Bcl-xS, Bak, Bid and Bad function conversely (Merry *et al.* 1997). Bcl-2 protein is predominantly located within the intracellular membranes and it protects both

the neural cell line and primary mouse cortical cultures from apoptotic and necrotic insults (Kane *et al.* 1993). Furthermore, overexpression of the Bcl-2 gene in transgenic mice reduced the infarct volume after focal cerebral ischaemia while Bcl-2 knockout mice showed the opposite effects in response to the same insult (Martinou *et al.* 1994; Hata *et al.* 1999).

1.2.5 Treatment for cerebral ischaemia

Current clinical treatment for cerebral ischaemia is limited and generally relies on rehabilitation and palliative care. The only licensed drug by the US Food and Drug Administration (FDA) for treating cerebral ischaemic patients is recombinant tissue plasminogen activator (rt-PA), aimed at dissolving the thrombus to allow restoration of blood flow. Utilization of rt-PA improves outcomes (Hacke *et al.* 2004) while intravenous administration of rt-PA is only available to a small proportion of patients, due to the short treatment time window (3 hours after onset of ischaemia) (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group 1995), risk of haemorrhage (Warlow *et al.* 1997) and potential neurotoxicity (Kaur *et al.* 2004; Goto *et al.* 2007).

1.2.5.1 Failed neurprotectants in clinical trials

Gradual unravelling of the ischemic cascades has introduced the concept of neuroprotection. Based on previous research, blockade of one or more factors in death pathways, or overwhelming activation of survival pathways can lead to the protection of neuronal cells from damage or death (Green *et al.* 2006). The first strategy has undoubtedly become the priority for the development of neuroprotectants, for the mechanisms of ischemic cell death in cerebral ischaemia have now been well elucidated. Therefore, numerous inhibitors or blockers of ion

channels, antagonists of excitotoxic factors and scavengers of free radicals have been developed and tested in pre-clinical trials, most of which achieved great success at various levels from *in vitro* single cell, cell culture, brain slices to *in vivo* animal models (Green *et al.* 2006; Hossmann 2006). However, these pre-clinical data are poorly translated into clinical trials. Systematic review on 1026 interventions, which covers a wide range of experimental treatments in acute stroke, has revealed that only rt-PA, aspirin and stroke unit care are effective in clinical trials (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group1995; International Stroke Trial Collaborative Group 1997; O'Collins *et al.* 2006). Clearly, none of these interventions are related to neuroprotection per se. Whether such neuroprotective drugs exist and can be useful remains to be identified. There are several reasons to explain the poor translation of candidate drugs to the clinic implications as highlighted by systematic review analysis, which include improper experimental design, false positive results from animal studies and different responses to ischaemia between animal and human brains (Sena *et al.* 2007; Shuaib *et al.* 2008). More importantly, the strategy based on blocking death signalling requires a closer examination particularly given the most recent drug failure at clinical trial. NXY-059, a free radical trapping agent considered to be highly promising and has proved effective in pre-clinical experiments following all rigorous STAIR (Stroke Therapy Academic Industry Round Table) criteria (Zhao *et al.* 2001; Sydserff *et al.* 2002; Green *et al.* 2003; Wang *et al.* 2004), failed in clinical trials (Shuaib *et al.* 2007). This adds more concerns on the strategy for drug development. Table 1 gives detailed information of some neuroprotective agents in clinical trials and their outcome illustrates the concern over the effectiveness of so called neuroprotective drugs for cerebral ischaemia.

Table 1-1 Summary of failed trials of neuroprotective agents

Product	Mechanism	Outcome	Comments
Nimodipine	Calcium channel blocker	Mixed effects on outcome	Blood pressure affects outcome (Fogelholm *et al.* 2004)
Fosphenytoin	Sodium channel blocker	Phase III trails failed	Lack of efficacy
BMS-204352	Potassium channel opener	Phase III trails failed	
Selfotel	NMDA antagonist	No efficacy in Phase III trials	Poorly tolerated with a potential neurotoxicity (Davis *et al.* 2000)
Aptiganel	NMDA channel blocker	No efficacy in Phase III trials	Potential detrimental effects in patients (Albers *et al.* 2001)
Gavestinel	Glycine antagonist	No efficacy in Phase III trials	
Tirilazad	Lipid peroxidation inhibitor	Outcome is worsned	Only works in reperfusion model
Lubeluzole	Ion channel and nitric oxide blocker	No efficacy in Phase III trials	Potential increase in heart conductance disorders (Gandolfo *et al.* 2002)
UK-279,276	Neutrophil inhibitory factor	Phase III trails failed	Only work in reperfusion models
Trafermin	Growth factor	Phase II/III trails halt	Lack of efficacy
Repinotan	5-HT_{1A} receptor antagonist	Phase IIb trial halt	Disappointing results
ONO-2506	Astrocyte modulating agent	Unfavourable interim analysis of Phase II	

This table is modified from. (Beresford *et al.* 2003; Shuaib *et al.* 2008)

1.2.5.2 Hypothermia

Recently the endogenous protective mechanisms have become the central stage in the research on neuroprotection following cerebral ischaemia (Dirnagl *et al.* 2003). Boosting such mechanisms provides an alternative strategy for potential neuroprotectant development.

As a natural mechanism which is exploited by many mammalian species to adapt to extreme environment, hypothermia is known to be neuroprotective for a long time. A recent systematic review analysis on animal models of focal cerebral ischaemia showed that outcome in the hypothermic treatment group was significantly improved by approximately 30% (van der Worp *et al.* 2007). The first attempt to use hypothermia as a clinical treatment was back to 1940s for the treatment of severe traumatic brain injury (Fay 1959). With accumulated evidence, hypothermia is evaluated in randomized clinical trials in patients with global cerebral ischaemia after cardiac arrest (Hypothermia after Cardiac Arrest Study Group 2002; Bernard *et al.* 2002) and in infants with hypoxic-ischaemic encephalopathy (Shankaran *et al.* 2005), which confirms cooling is indeed beneficial for the reduction of mortality and morbidity.

In general, two types of hypothermia have been defined: mild (32~35°C) and moderate hypothermia (30~33°C) (Gentilello 1995). Previous studies in transient global ischaemia showed that long-term moderate hypothermia provided sustained behavioural and histological neuroprotection (Busto *et al.* 1989; Buchan *et al.* 1990; Baker *et al.* 1995), while brief and mild hypothermia only delayed the neuronal damage (Dietrich *et al.* 1990). Hypothermia performed soon after the onset of ischaemia and maintained for an adequate length of time is well known to be neuroprotective. However, hypothermia delayed up to 2 hours after onset of focal ischaemia has also shown benefit, provided that it was maintained for at least

1–3 hours (Young *et al.* 1983). In transient global ischaemia, mild hypothermia performed for 3 hours at reperfusion onset was very effective at reducing neuronal damage 3 days after ischaemia (Dietrich *et al.* 1990). Although current evidence suggests a minor delay in hypothermia up to 3 hours is still beneficial for neuronal survival, it makes sense to begin the hypothermic treatment as soon as possible after the ischaemic onset. Except for the time-window, the effectiveness is also largely determined by other factors such as duration and depth of hypothermia, types and severity of ischaemia.

Although the mechanisms of hypothermia in neuroprotection are not entirely clear, it has been suggested that they are associated with energy reduction (Williams *et al.* 1994), maintenance of blood-brain barrier stability (Jiang *et al.* 1992), inhibition of excitotoxic glutamate release (Busto *et al.* 1989), suppression of free radical generation (Gordon 2001) and up-regulation of anti-apoptotic genes, such as Bcl-2 (Zhang *et al.* 2001) and neurotrophic factors (D'Cruz *et al.* 2002). In addition, hypothermia also triggers the endogenous signalling pathways PI3K/Akt (Zhao *et al.* 2005) and MAPK (Hicks *et al.* 2000) during cerebral ischaemia to affect the downstream gene expressions, altering the balance between survival and death in cell fate.

However, there are several limitations to the utilization of hypothermia in cerebral ischaemia. The methods of hypothermia, particularly depth, duration, rewarming and medications need to be assessed and optimized for individual ischaemic patient. Therefore it may significantly delay the time of application in clinical usage. Hypothermia also causes some side-effects in clinical treatment. In 50 prospective patients with cerebral infarction, moderate hypothermia caused thrombocytopenia (70%), bradycardia (62%) and pneumonia (48%) and 15 patients (30%) died during or after rewarming because of rebound increase in

intracranial pressure (Schwab *et al.* 2001). The other side-effects include thermoregulatory shivering (Frank *et al.* 1993) , myocardial infarction and infection (Krieger *et al.* 2001).

Although much evidence shows that hypothermia is a potent neuroprotectant against cerebral ischaemia, it is still difficult to deliver clinically. Identifying the mechanism(s) by which hypothermia exerts its neuroprotective effect and using this instead may prove a more suitable target for future therapeutic strategies.

1.3 Corticosteroids

Corticosteroid hormones are a class of steroid hormones made in the adrenal gland which are essential for life and required for several physiological systems in mammals. Corticosteroids can be subdivided into two groups, glucocorticoids (GC) and mineralocorticoids. The main naturally occurring GC is cortisol in humans and corticosterone in rodents, and the main mineralocorticoid is aldosterone. Under physiological conditions, the level of GC is approximately three orders of magnitude higher than the aldosterone.

1.3.1 Function

The primary physiological function of aldosterone is the control of electrolyte homeostasis and blood pressure. The major site of action is the kidney where aldosterone promotes renal sodium reabsorption and potassium secretion in the distal nephron. Other aldosterone targets include the distal colon, salivary and sweat glands where the primary function is to maintain the electrolyte balance. One consequence of electrolyte reabsorption is the effect on the blood pressure,

since the water is reabsorbed thereby the intravascular volume is increased, which subsequently has an influence on blood pressure. Aldosterone also has actions at non-mineralocorticoid target tissues including the heart, vascular system and the brain. Prolonged aldosterone exposure in these systems can have detrimental consequences promoting vascular remodelling, cardiac fibrosis, and hypertension (Gomez-Sanchez 2004).

In comparison to aldosterone, GCs has more effects on a wide range of physiological functions in the body including those required for energy balance and metabolism, immune function, cardiovascular function, circadian rhythmicity, growth and development, cognitive processes and the expression of various types of behaviour. For example, GCs regulate energy storage by participating in glycogenic and glycolytic pathways (Villar-Palasi *et al.* 1970; Nyirenda *et al.* 1998). GCs are also a powerful immunosuppressant, which led to the development of therapeutic applications for the treatment of asthma (Stellato 2007) and rheumatoid arthritis (Gaffo *et al.* 2006). In foetal development, GCs promote the maturation of the lungs and are involved in the differentiation of neural crest cells (Anderson 1993; Garbrecht *et al.* 2006).

A major target of GCs is the central nervous system where they exert a broad range of effects. The hippocampus, an important region for mood, memory and behaviour, is one of the primary targets of GC action. Both learning and memory formation have been demonstrated to be closely associated with GC action (Bohus *et al.* 1981; Oitzl *et al.* 1992; McEwen 2005; de Kloet *et al.* 2008). GCs are also involved in the coordination of circadian events as the sleep-awake cycle and food intake (Dallman *et al.* 1995; Bradbury *et al.* 1998). The mechanisms may be due to the effects of GCs on neurotransmitter systems and neuronal excitability (Joels 1997). In the hippocampus, some of neurotransmitter factors are extremely

sensitive to the level of circulating GCs, including the excitatory effects evoked by β-adrenoceptor activation (Joels *et al.* 1989), the hyperpolarization mediated by serotonin-1A receptor (Joels *et al.* 1991) and the Ca^{2+} influx by the activation of voltage-dependent calcium channels (Kerr *et al.* 1992). Long-term potentiation (LTP), a prolonged enhancement of synaptic contacts, which is believed to contribute to learning and memory formation (Lynch 2004), is also affected by GCs (Kim *et al.* 2002).

In addition to behavioural effects, GCs are also known to play an important role in maintaining neuronal integrity, enhancing neurogenesis and regulating neuronal survival (Sapolsky 1996; Abraham *et al.* 2006).

1.3.2 Synthesis and regulation of corticosteroids

Endogenous corticosteroids are synthesized in the adrenal cortex from the common precursor cholesterol through a series of enzymatic reactions. Because of the wide-ranging actions of GCs, circulating levels must be tightly regulated. The neuro-endocrine system that controls GC secretion is known as a negative feedback loop hypothalamus-pituitary-adrenal gland (HPA) axis (Aguilera 1998).

The HPA axis is activated either by a circadian signal or by a stress (either physical or psychological) stimulus. Neural inputs which perceive these signals converge at the main HPA axis control centre, the hypothalamus to initiate secretion. Under non-stress conditions, circulating levels of GCs are released both in an ultradian and diurnal manner. The circadian rhythm is driven by an endogenous biological 'clock' in the suprachiasmatic nucleus and the rhythm is set by recurring daylight and darkness (Takahashi *et al.* 1982). In mammals, the peak secretion of GCs occurs just before the active period, whereas the nadir occurs

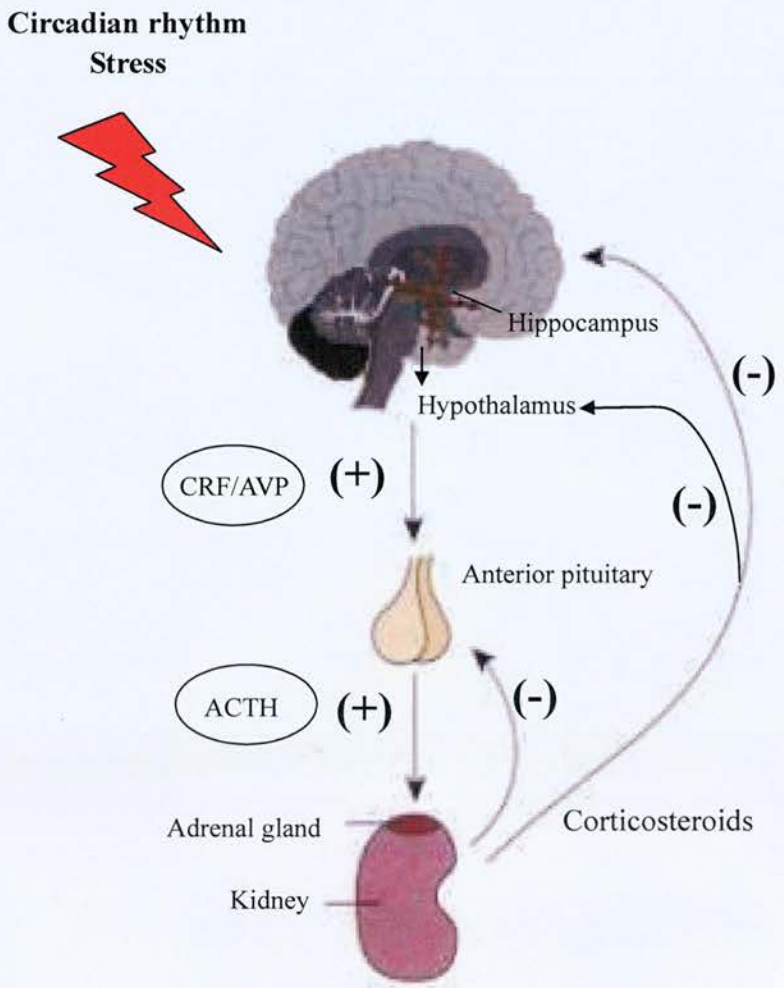


Fig 1-3. The regulation of GC by a negative feedback loop HPA axis. Stimuli augment the secretion of CRH/AVP and subsequently ACTH. This induces GC synthesis, which exerts its biological functions. An increase in concentrations of GCs executes feedback inhibition on HPA axis in pituitary and hypothalamus, reducing the synthesis of GC in the adrenal gland to restore the normal level of circulating GC. Refer to text for details.

during the rest period.

In response to a stimulus, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are rapidly released from the parvocellular cells of the paraventricular (PVN) nucleus of hypothalamus into the portal circulation to target the anterior pituitary. Here CRH and AVP act synergistically to stimulate the secretion of stored adrenocorticotropic hormone (ACTH) from corticotropic pituitary cells into the bloodstream. Circulating ACTH binds to its receptor located at the cell surface of the adrenal cortex to signal an increase in the synthesis of GCs from cholesterol. Once synthesized, GCs are immediately released into the systemic circulation to act throughout the body.

At the same time secreted GCs exert a negative feedback on the HPA axis by suppressing CRH in the hypothalamus and ACTH in the pituitary (Ma *et al.* 1997), to prevent further synthesis of GC in the adrenal gland and restore the normal basal levels (Dallman 2005). This process is critical, because sustained high level of GCs causes a series of harmful effects in the body.

1.3.3 Bi-directional effect of GCs on neuronal viability

The maintenance of normal neuronal integrity and function is dependent on physiological concentrations of GCs (Abraham *et al.* 1997). Either significantly reduced or chronically increased GC levels can directly threaten neuronal viability. For example, a lack of GCs (e.g. following adrenalectomy) results in loss of granule cells of the hippocampal dentate gyrus (DG) in adult rats by apoptosis (Sloviter *et al.* 1989; Sloviter *et al.* 1993) while overexposure to GCs enhances neuronal vulnerability to toxic challenge particularly in the pyramidal cells of the CA3 region of the hippocampus (Landfield *et al.* 1978; Kerr *et al.* 1991; Hibberd

et al. 2000).

It is worth mentioning that aberrant concentrations of GCs do not induce neuronal apoptosis directly but endanger the cells by enhancing their vulnerability to neurotoxic insults, such as cerebral ischaemia. It has been shown that elevated plasma GC concentrations following cerebral ischaemia were associated with increased morbidity and mortality in humans (Feibel *et al.* 1977; Olsson 1990; Fassbender *et al.* 1994) and increased infarct size in mice (DeVries *et al.* 2001). Other evidence from animal models also suggests a link between GC concentrations and focal cerebral ischaemic outcome, in which the manipulation of GC levels prior or post-focal cerebral ischaemia affects the infarct size (Sapolsky *et al.* 1985; Morse *et al.* 1990; Rami *et al.* 1998; DeVries *et al.* 2001; May *et al.* 2002). Similarly, in global cerebral ischaemia models, exposure to high concentrations of GCs also compromises neuronal viability in the hippocampus (Kofler *et al.* 2004; Neigh *et al.* 2004; Neigh *et al.* 2005).

The major mechanisms through which GCs endanger neuronal cells in exposure to toxic insults include inhibition of the uptake of key nutrients such as glucose (Doyle *et al.* 1993; Sapolsky 1996), modulation of both excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission (Moghaddam *et al.* 1994; Abraham *et al.* 1996) and direct enhancement of intracellular Ca^{2+} signalling (Kerr *et al.* 1989; Joels *et al.* 1994).

1.4 The corticosteroid receptors

The actions of corticosteroids are mediated via binding to two structurally related intracellular receptors: the mineralocorticoid (MR) and glucocorticoid receptor

(GR). Both receptors act as transcriptional factors to alter the transcription of both overlapping and distinct target genes (Datson *et al.* 2008).

1.4.1 Distribution

MR and GR differ in their distribution. GR is ubiquitously expressed in almost every cell in the body. Within the brain, GR has a wide distribution in both neurons and glia, and is highly expressed in the limbic system, particularly the hippocampus, hypothalamus, and the supraoptic nucleus (de Kloet *et al.* 2000). GR is also present in relatively high concentrations in the ascending monoaminergic neurons of the brain stem (Kerr *et al.* 1989; Joels *et al.* 1994).

Compared to GR, MR has more specific distribution which is generally divided into epithelial and non-epithelial tissues. In epithelial cells of the kidney, MR expression is located in the distal convoluted tubules and cortical collecting ducts (Krozowski *et al.* 1989; Lombes *et al.* 1990). It is also found in glomeruli especially in mesangial cells (Nishiyama *et al.* 2005) and podocytes (Shibata *et al.* 2007). MR expression in other epithelial tissues include the distal colon (Pressley *et al.* 1975; Lombes *et al.* 1984; Rafestin-Oblin *et al.* 1989), lung (Krozowski *et al.* 1981), salivary glands (Pressley *et al.* 1975; Lombes *et al.* 1984; Rafestin-Oblin *et al.* 1989), sweat glands (Funder *et al.* 1972), inner ear (Trune *et al.* 2006) and skin (Kenouch *et al.* 1994).

In non-epithelial tissue, MR is found in mononuclear leucocytes (Armanini *et al.* 1985), cardiomyocytes of the heart (Barnett *et al.* 1988) and in both white and brown adipose tissue (Zennaro *et al.* 1998; Caprio *et al.* 2007). Other sites of MR expression include the retina (Mirshahi *et al.* 1997), placenta (Hirasawa *et al.* 2000) and reproductive tissues including the uterus, ovaries and testis (Le Menuet

et al. 2000).

In comparison to GR, the expression of MR is limited to specific regions of the central nervous system. The highest levels of MR are found in the limbic system – particularly in the hippocampus – with lower expression in other limbic structures such as the lateral septum and amygdale. In addition, MR is also expressed in the olfactory nucleus, layer II of the cortex and in brainstem sensory and motor neurons (Arriza *et al.* 1988; Herman 1993; Reul *et al.* 2000; Kretz *et al.* 2001). Low levels of MR expression are also found in the anterior hypothalamus, subfornical organ and choroid plexus (Van Eekelen *et al.* 1988). Within the hippocampus, MR distribution overlaps with GR in the pyramidal cells of the CA1 and CA2 subregions and in the granule cells of the dentate gyrus (Ito *et al.* 2000; Han *et al.* 2005). In CA3 and CA4 regions, MR expression is considerably higher than GR in both the adult and the neonatal rats (Avishai-Eliner *et al.* 2001; Jongen-Relo *et al.* 2002).

1.4.2 Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) signalling

In the absence of ligand, GR and MR are primarily located in the cytoplasm bound in a complex with heat shock protein (HSP)-70 and HSP-90 chaperone proteins (Pratt *et al.* 2004) and with immunophilins such as FKBP51 (Zhou *et al.* 2005). The association of the receptors with HSPs maintains the receptor in a conformation that permits high-affinity ligand binding (Picard *et al.* 1990). Following ligand binding, the receptor undergoes a series of conformational changes, leading to the detachment of the chaperone proteins which exposes the nuclear localization signal. These signals recruit a group of nuclear translocation proteins (importins) that actively shuttle the ligand bound receptor into the nucleus

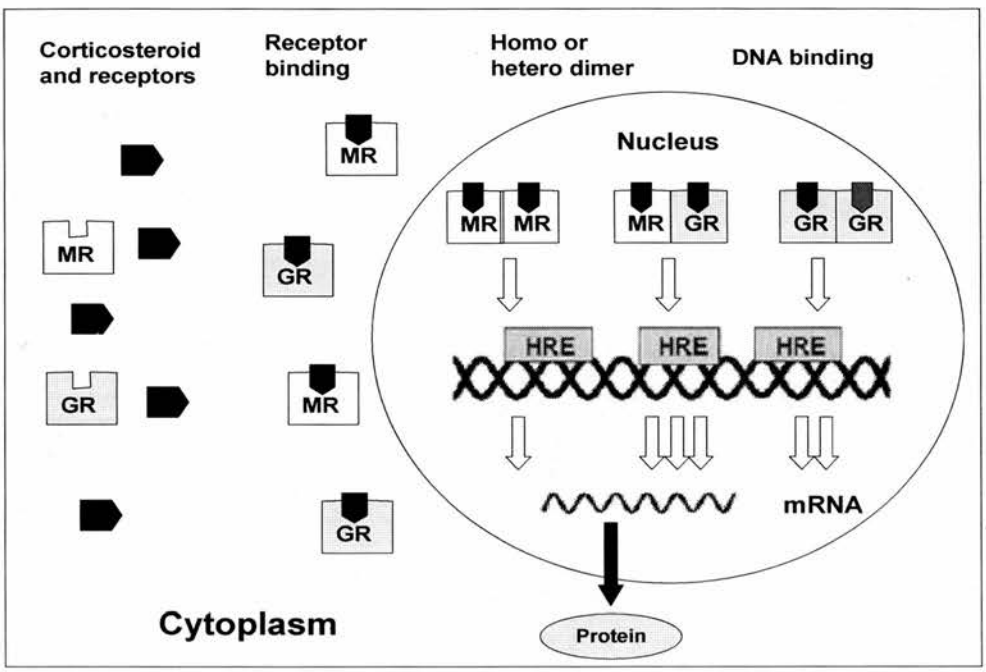


Fig 1-4 Schematic illustration of the activation of corticosteroid receptors. MR and GR dissociate from the chaperone complexes upon ligand binding and are translocated to the nucleus where they form homodimers or heterdimers. These dimers bind to Glucocorticoid response elements (GRE) of the target genes to modulate their expression. Refer to text for details

(Isohashi *et al.* 1993; Zhou *et al.* 2005).

Most GR- and MR-mediated gene activation requires dimerization of its receptors as either homo- or hetero-dimers (ie GR/GR, MR/MR or GR/MR) (Forman *et al.* 1995; Liu *et al.* 1995) (Fig 1-4). Formation of heterodimers has been suggested to provide a more finely orchestrated regulation of corticosteroid-responsive genes than the actions based on homodimerization alone (Trapp *et al.* 1994; Trapp *et al.* 1996). Homodimeric MR/MR and GR/GR or heterodimeric MR/GR bind to a specific palindromic DNA sequence known as the glucocorticoid response element (GRE), which is located within the enhancer and/or promoter region of the target genes (Beato *et al.* 1995; So *et al.* 2007). In doing so, this causes chromatin remodelling and enables the recruitment of components of the basic transcriptional machinery to allow RNA synthesis (Beato *et al.* 1995). Fig1-4 shows a schematic representation of trans-activation of GR and MR. Glucocorticoid-response element (GRE: 5'-GGTACAnnnTGTt/cCT-3') has been well elucidated and shown to be variable extensively around a consensus, but is strikingly conservative for a given site across species for both GR and MR (Beato 1989; Alnemri *et al.* 1991; Tsai *et al.* 1994). However, the existence of an MR-specific mineralocorticoid-response element (MRE) is still a matter of controversy. Kolla described a response element designated as (MRE/GRE) in human Na/K ATPase α1 promoter which shows greater preference to MR than GR (Kolla *et al.* 2000), suggesting the existence of an MRE.

In addition to trans-activation, GR and MR can also repress the transcription of some specific genes. For example, expression of 5-HT_{1A} receptor gene in hippocampus is negatively regulated by MR (Nishi *et al.* 1996; Meijer *et al.* 1997) and/or MR/GR heterodimers (Ou *et al.* 2001). The mechanism of such trans-repression is similar to trans-activation, which involves the homodimers or

heterodimers of MR and GR bound to a specific DNA element in the promoter region of the target gene. This specific DNA element is known as negative GRE (nGRE). An nGRE shows a highly variable sequence in comparison to GRE (Truss *et al.* 1993; de Kloet *et al.* 2000). Upon binding to nGRE, corticosteroid receptors can either inhibit the gene expression directly (Aumais *et al.* 1996) or occlude adjacent or overlapping binding sites for those positively acting transcriptional factors, thereby eliciting a negative response indirectly (Schoneveld *et al.* 2004). Remarkably, a second mechanism has been described for GR-mediated trans-repression that binding of the receptor dimers to nGRE is no longer required. This is achieved by modulating the activity of the other transcriptional factors or co-activators through protein-protein interactions (de Kloet *et al.* 2000; Schoneveld *et al.* 2004).

1.4.3 Ligand binding

1.4.3.1 Affinity

GR and MR differ in their affinity for corticosteroids. Although GC is the primary hormone for GR activation, the affinity of GR for GCs (K_d=1–5nM) is approximately 10 fold lower than MR (K_d=0.3nM) (Reul *et al.* 1985). However, GR displays a much higher affinity for synthetic glucocorticoids such as dexamethasone (DEX) (Reul *et al.* 1987b) than its endogenous ligand. MR exhibits a similar high affinity for both aldosterone and GC in a variety of species including rat, mouse, hamster, dog and human (De Kloet *et al.* 1975; Veldhuis *et al.* 1982; Reul *et al.* 1985; Rupprecht *et al.* 1993).

1.4.3.2 11β-HSD1 and 11β-HSD2

Although MR binds GC and aldosterone at equal affinity, it selectively bind aldosterone in the face of up to 1000-fold molar excess of circulating GCs in the epithelial cells of the kidney, colon, sweat glands which regulate fluid volume and electrolyte homeostasis (Seckl 1997). This specificity of MR to aldosterone is dependent on the presence of an enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Edwards *et al.* 1988; Funder *et al.* 1988). At pre-receptor level, 11β-HSD2 converts corticosterone or cortisol to the inert 11-keto forms so that they can not bind to MR. Therefore, aldosterone at much lower concentration can access the receptor and manifest its biological effects (White *et al.* 1995). This enzyme is primarily found in the aldosterone target tissues such as distal nephron of kidney (Moisan *et al.* 1992), colon (Brown *et al.* 1996), placenta (Brown *et al.* 1996) and vascular endothelium (Hadoke *et al.* 2001). In the brain, it is expressed in a few and discrete nuclei implicated in mediating central control of salt/water balance and blood pressure by aldosterone (Roland *et al.* 1995; Robson *et al.* 1998).

In addition, there exits another type of enzyme termed as 11β-HSD1 (11β-hydroxysteroid dehydrogenase type 1) which has an opposite effect in comparison to 11β-HSD2. 11β-HSD1 is capable of regenerating GCs from their 11-keto forms and expressed in a variety of tissues such as liver (Agarwal *et al.* 1989), lung (Tannin *et al.* 1991) but absent in aldosterone target cells (Shackleton *et al.* 1985; Stewart *et al.* 1987). In the brain, the expression of 11β-HSD1 is found in cerebellum, hippocampus, neocortex, hypothalamus and anterior pituitary (Seckl *et al.* 1993; Seckl 1997). The function of 11β-HSD1 is to potentiate the GC signals and effects on regulation of HPA axis (Kotelevtsev *et al.* 1997; Harris *et al.* 2001), neuronal survival (Rajan *et al.* 1995) and stress responses (Harris *et al.* 2001).

1.4.3.3 U-shape dose dependency of MR and GR in the brain

Crucially the brain contains little 11β-HSD2 and 11β-HSD1 is expressed in the hippocampus (Seckl 1997), so MR is predominantly bound to GCs. Because of the difference in affinity between GR and MR for the ligand, MR is approximately 80%-90% saturated under basal levels of GCs, such as at circadian nadir (Reul *et al.* 1987). In contrast, the occupancy of GR is quite low at basal level of circulating GCs (Reul *et al.* 1985) and activation of GR requires increased GC levels in the events such as the circadian peak or a stressful stimulus (Reul *et al.* 1987a; Reul *et al.* 1987b). Therefore the cellular responses to GCs in the hippocampus manifests a U-shape dose-response relationship with plasma corticosteroids (Diamond *et al.* 1992; Joels *et al.* 1994), for example, the amplitude of depolarisation-induced calcium currents (Joels *et al.* 2003), the hyperpolarization cased by serotonin-1A receptor activation (Joels *et al.* 1991; Hesen *et al.* 1996) and glucocorticoid-mediated neuronal vulnerability to toxic insults (Abraham *et al.* 2006).

1.4.4 GR and MR in HPA axis

The main function of GR in the brain is to restore the basal activity of the HPA axis at the hippocampal, hypothalamic and the pituitary levels after stressful stimuli or during the diurnal peak. Lack of GR function impairs the negative feedback of GC, in which HPA axis activity is sustained causing chronically high plasma concentrations of CRH, ACTH and GC (Hesen *et al.* 1996; Kretz *et al.* 1999). On the other hand, overexpression of GR in transgenic mice shows lowered circulating GC levels due to increased sensitivity to the ligand in the hypothalamus, which suppresses the synthesis of CRH and AVP and subsequently GC synthesis in the adrenals (Reichardt *et al.* 2000).

In the brain MR is occupied to a large extent in the limbic neurons under normal conditions, which raised the question why constant activation of MR is required. It was first proposed that MR activation is involved in the tonic inhibitory control of the hippocampus on HPA activity (De Kloet *et al.* 1987). This concept was later tested by various studies using MR antagonist in the animal models. Intra--cerebroventricular and intra-hippocampal injection of the synthetic MR antagonist RU28318 results in an elevation of basal GC levels (Oitzl *et al.* 1995). Rats treated with RU28318 or with antisense MR mRNA show increased HPA response to psychological stressors (Ratka *et al.* 1989; Reul *et al.* 1997). Aged dogs, which generally have enhanced responses to stress, showed decreased septal and hippocampal MR expression and unaltered GR, accompanied by elevated basal plasma ACTH and GC levels (Reul *et al.* 1991; Rothuizen *et al.* 1993) Therefore, hippocampal MR enhances inhibitory tone on basal and stress-induced HPA release in rodents and human (Reul *et al.* 2000).

1.4.5 Opposing effects of GR and MR in neuronal cell fate

Previous studies suggest that different activation of the dual receptor system, which is formed by MR and GR, may account for the opposing actions of corticosterone on neuron proliferation, survival, and death in the brain, specifically in hippocampal subfield and dentate gyrus (Gould *et al.* 1996; Hassan *et al.* 1996; Reagan *et al.* 1997).

1.4.5.1 GR in neuronal death

Under stress conditions, increased GR occupation leads to the arrest of neurogenesis in the dentate gyrus (Gould *et al.* 1998) and at the same time

stimulates apoptosis within the granular and hilar cell populations of the dentate gyrus (Hassan *et al.* 1996). Experiments using specific GR agonist DEX in primary hippocampal culture *in vitro* and in rats *in vivo* showed that DEX significantly increased cell death (Crochemore *et al.* 2005). More importantly, GR activation has been linked to deteriorating neuronal damage produced by hypoxia-ischaemia insults (Koide *et al.* 1986; Packan *et al.* 1990; Adachi *et al.* 1998). A surging elevation of GC levels was induced shortly after the ischaemic insult, which is believed to saturate GR and contributed to the increased cell death (DeVries *et al.* 2001; Neigh *et al.* 2005). Adrenalectomized animals following ischaemic episode had attenuated neuronal loss in hippocampus (Sapolsky *et al.* 1985), whereas this protective effect was removed by chronic treatment with DEX (Koide *et al.* 1986). Furthermore, the administration of selective GR antagonist RU38486 in adult rats significantly protected hippocampal CA1 pyramidal cells from transient cerebral ischaemia (Antonawich *et al.* 1999). The mechanisms of this GR-induced neuronal endangerment are not entirely clear, but implied to be associated with energy-dependent elevation of extracellular glutamate concentrations and subsequently the increased intracellular Ca^{2+} influx (Sapolsky 1986; Virgin *et al.* 1991; Elliott *et al.* 1993; Adachi *et al.* 1998). It has also been suggested by Almeida that cell death due to GR activation was attributable to the up-regulation of the pro-apoptotic gene, Bax, and its direct transcriptional regulator, p53 (Almeida *et al.* 2000).

1.4.5.2 MR in neuroprotection

MR signalling is essential for cell survival. Animals adrenalectomized for a period of 3–7 days show a substantial decrease in cell number in hippocampal dentate gyrus (Gould *et al.* 1990). However, replacement with aldosterone in those animals protected the cells from death but DEX had no effects, confirming MR

not GR activation is required for neuronal survival (Woolley *et al.* 1991; Sloviter *et al.* 1995). Further evidence came from MR knockout mice, which showed impaired neurogenesis and degeneration of the hippocampal granule cells in adulthood (Gass *et al.* 2000). In primary hippocampal culture, DEX-induced cell death was rescued by treatment with aldosterone and this protective effect was abolished by the MR antagonist (Crochemore *et al.* 2005). Taken these together, it suggests MR activation at physiological concentrations of GCs plays a vital role in maintaining the neuronal viability and hippocampal integrity and counteracting GR-induced cell death.

Our group has shown that MR mRNA and protein are rapidly increased in rat primary cortical and hippocampal cultures in response to the general-apoptosis inducer staurosporine. This increase in MR was associated with enhanced survival since MR antagonism with spironolactone removed this protective effect (Macleod *et al.* 2003). MR induction was also observed *in vivo* in rat hippocampus after exposure to hypothermic transient global ischaemia. Similarly, the administration of spironolactone promoted cell death (Macleod *et al.* 2003). Importantly, overexpression of MR in PC12 cells protected against cell death caused by staurosporine and by oxygen-glucose deprivation (*in vitro* ischaemic insult). This survival effect was again abrogated by MR antagonism (Lai *et al.* 2005) and similarly *in vivo*, transgenic mice overexpressing MR specifically in the forebrain displayed protected against the damaging effects of transient global cerebral ischaemia (Lai *et al.* 2007).

Increased MR expression was only found in the hypothermic ischaemic group but not in hypothermic or ischaemic groups alone, indicating that MR may be involved in endogenous survival pathways triggered by hypothermia and cerebral ischaemia. Although the mechanisms of MR in neuroprotection are not entirely

clear, evidence suggests MR activation leads to overall decreased NMDA receptor activity followed by reduced calcium influx (Nair *et al.* 1998). In addition, MR signalling promotes the expression of the anti-apoptotic protein Bcl-2 and Bcl-xl (Almeida *et al.* 2000; McCullers *et al.* 2001) as well as basic fibroblast growth factor and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) (Hansson *et al.* 2000).

Interestingly MR mRNA levels have recently been found to be increased in the hippocampal neurons in human post-mortem brains from patients who suffered from transient global ischaemia due to cardiac arrest several days before their death (Lai M, Macleod M, in press), implying that a similar injury-induced MR neuroprotective mechanism exists in the human brain. Therefore manipulation of MR may provide a novel treatment strategy for conditions involving cerebral ischaemia.

1.4.6 Challenges to developing MR-based neuroprotective agents

Given the body of evidence indicating the beneficial effects of MR on neuronal survival, manipulation of MR signalling therefore appears to be a possible strategy for treating conditions involving cerebral ischaemia. Importantly, up-regulation of MR must be specific to neurons and not other types of cells. Since MR is almost saturated at basal level of circulating GCs, increasing MR signalling will require increased abundance of receptor instead of ligand concentration.

1.4.6.1 MR expression in glial cells

Except for neurons, MR is also expressed in glial cells such as astrocytes and oligodendrocytes (Chou *et al.* 1991; Cintra *et al.* 1994). Since glia cells provide

nutritional support, regulate the chemical environment, maintain the structural integrity and an immune defence system to the neurons in the CNS (Ransom *et al.* 2003; Volterra *et al.* 2005; Haydon *et al.* 2006), if MR signalling can protect glial cells from ischemic insult, it is reasonable to predict the MR up-regulation in glial cells will also be beneficial for neuronal survival in an indirect manner. However, few direct studies on the function of MR activation in glial cells in response to cerebral ischemia are available except one, which was reported by Hwang, who measured MR expression in astrocytes in response to transient global ischemia in gerbil and demonstrated an increased expression of the MR but failed to measure neuronal or glial viability (Hwang *et al.* 2006). Indirect evidence showed hypoxia-induced cell death was enhanced by additional GCs treatment in astrocyte culture *in vitro* whereas this deleterious effect is believed to be associated with GR activation instead of MR solely (Tombaugh *et al.* 1993). Therefore the function of MR activation in glial cells is still a mystery. In terms of drug development, it is more realistic and promising to focus on the augment of MR signalling in neurons, which is confirmed to exert protective function directly.

1.4.6.2 MR expression in vascular system

Activation of MR in cerebral vessels causes vascular remodelling and eventually lead to hypertension which itself is a risk factor for ischaemic stroke. The first report by Conn *et al.* in the 1960s proposed a link between hypertension, stroke and elevated plasma aldosterone levels (Conn *et al.* 1964). This link was further consolidated by studies using inhibitors of the rennin-angiotensin- aldosterone system (RAAS), which conferred greater protection from stroke than a reduction in blood pressure alone (Hilleman *et al.* 2004; Sokol *et al.* 2004). This suggests that reduced aldosterone concentration and presumably reduced MR activation is at least part of the beneficial effects. Experiments in stroke-prone spontaneously

hypertensive rats revealed that MR activation in cerebral vessels resulted in a reduced lumen size without a significant change in wall thickness (Dorrance *et al.* 2006). Furthermore, administration of MR antagonist spironolactone in these animals significantly reduced damage caused by cerebral ischaemia by 50% (Dorrance *et al.* 2001).

Therefore, these findings in which increased MR signalling in different tissue and cell types can either be beneficial or detrimental to cerebral ischaemic outcome suggest that developing MR based treatments needs to be specifically targeted. In order to do this a thorough understanding of the transcriptional regulation of MR is crucial.

1.4.7 The transcriptional regulation of MR

It is now clear that the MR gene does not transcribe just for one unique mRNA, but rather for a number of variants, in a manner analogous to GR (Yudt *et al.* 2002). The mechanism involved in generating distinct MR variants is known as alternative splicing, which is believed to potentiate the diversity of protein or mRNA species from a single gene.

The rat MR gene gives rise to three distinct MR mRNA transcripts (MRα, β, γ) which differ in their 5'-untranslated regions but encode the same mature protein (Kwak *et al.* 1993). Each corresponding 5'-flanking region acts as a functional promoter allowing independent regulation of each mRNA variant. Similarly, the human MR gene generates two MR mRNA species controlled by two separate promoters (P1 and P2) which correspond to rat MRα and MRβ respectively (Zennaro *et al.* 1995; Zennaro *et al.* 1996). Both RNase protection assay and *in situ* hybridization show that MRα and MRβ transcripts are equivalently expressed

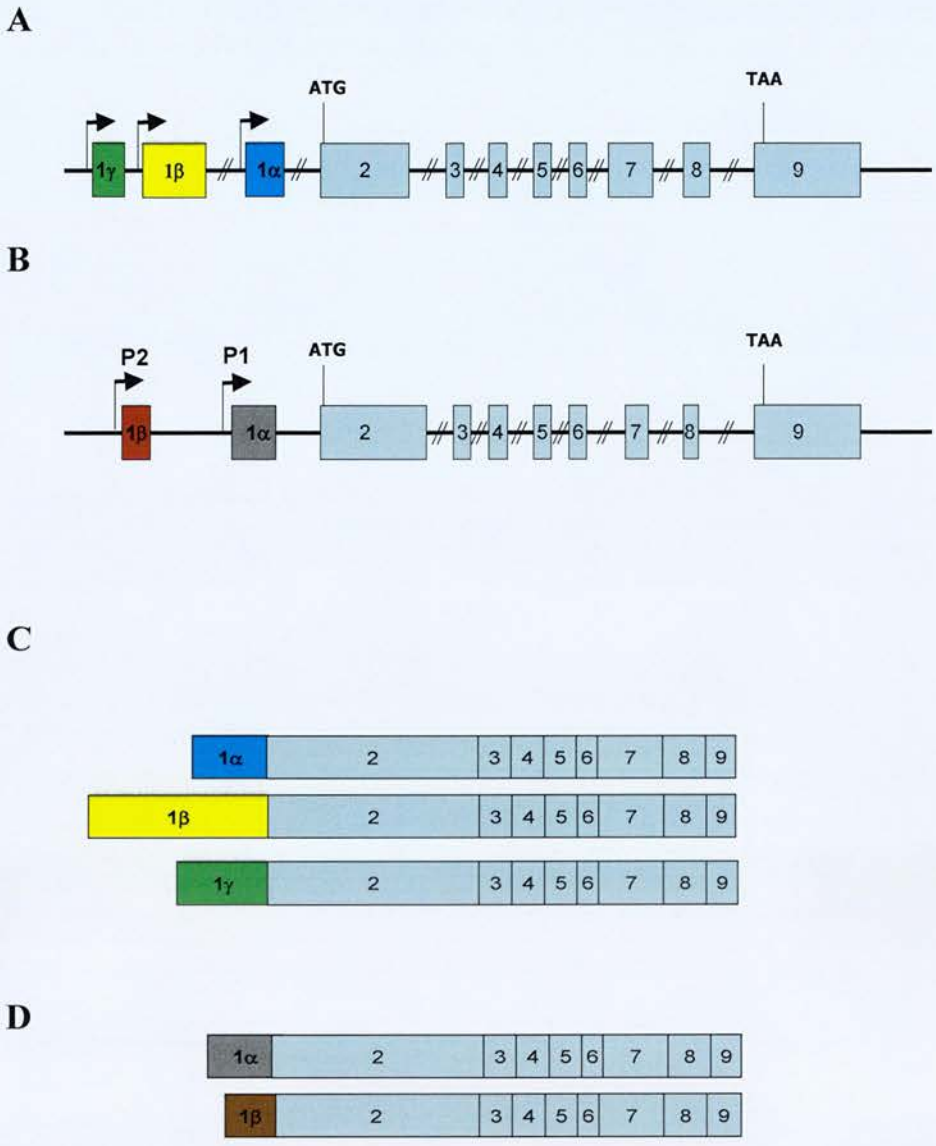


Fig 1-5 Schematic representation of MR gene in rat (A) and human (B) and mRNA variants in rat (C) and human (D). The rMR is composed of 11 exons, including three untranslated first exons (1α, 1β and 1γ) while the other exons encode the entire protein. The ATG translational start codon is located at 2bp of exon2, while the stop codon is located in exon9. Under the control of its own promoter, transcription of these 5'-untranslated exons generates three different mRNA variants, termed rMRα, rMRβ and rMRγ in rat. Human MR has two mRNA variants, termed hMRα and hMRβ, and transcription is directed by promoters P1 and P2 separately. TAA=stop codon.

in adult rat hippocampus, occupying 60% of total MR while MRγ is much less abundant (~ 3%). The ratio of transcripts however differ in different tissues, for example in the kidney the MRα variant is the only predominant variant and both rMRβ and rMRγ are poorly expressed (Kwak *et al.* 1993). The pattern of transcripts also depends on the stages of development: in the hippocampus of neonatal rat, rMRγ is highly expressed from postnatal day 3 to day 7 then declines rapidly; whereas, rMRα mRNA levels remain throughout development (Vazquez *et al.* 1998).

Little is known about how each variant MR transcript is regulated. To investigate this a 1500bp promoter fragment of the MRα variant was generated by Castren (Castren *et al.* 1993) and promoter activity was found to be up-regulated by corticosterone and progesterone (Castren *et al.* 1995; Castren *et al.* 1995a). Other regulators of MRα have yet to be identified and to date there are no reports on how rMRβ or rMRγ are regulated.

1.4.8 The translation of MR

Given the translation initiation site is located 2bp downstream from the beginning of exon2, all of MR mRNA variants encode the same protein product (Pascual-Le Tallec *et al.* 2005). However, there exist some MR protein variants as well, for example, a 12bp insertion at the exon3/intron splice junction leads to an inframe insertion of four amino acids in the DNA binding domain (Bloem *et al.* 1995) and this variant has no different in transcriptional activity in comparison to wild type MR (Bahr *et al.* 2004). Another protein variant is the 10bp deletion in the rat MR in the ligand binding domain, which is expressed at a low level and manifests unresponsiveness to aldosterone (Zhou *et al.* 2000). The reason for the MR protein diversity is still unclear, but it might contribute to a better modulation of overall

receptor functions.

1.5 Aims

Previous work suggests that MR is important in promoting neuronal survival for example in response to the damaging effects of cerebral ischaemia. How MR is regulated under such conditions has yet to be elucidated. Therefore identifying the exact mechanism should inform and guide the development of novel and effective MR-based treatment strategies for conditions causing cerebral ischaemia and other neurodegenerative diseases.

The overall aim of this thesis was to investigate in detail the transcriptional regulation of the rat MR gene in response to different cellular stressors. To accomplish this goal, investigations were carried out in three different models—in cell lines, primary neuronal cortical culture and in an *in vivo* model. Specifically, the aims were:

(i) To elucidate the transcriptional regulation of MR *in vitro* at the promoter level in response to different types of cell stress.

(ii) To demonstrate that altered promoter activity in response to cell stress corresponds to changes at the transcript level *in vitro.*

(iii) To establish if findings from (i) and (ii) are of biological relevance *in vivo.*

Chapter 2

MATERIALS AND METHODS

2.1 Gel analysis

2.1.1 Agarose gel electrophoresis

0.8% agarose gels were typically prepared for analyzing DNA fragments and plasmids by electrophoresis. Agarose was dissolved in 1x Tris/acetic acid/EDTA (TAE) buffer by boiling in a microwave oven. Ethidium bromide (final concentration 0.2ug/ml) was added to the molten agarose and cast in horizontal gel trays. Set gels were submerged in 1x TAE in standard gel electrophoresis tanks, then samples suspended in 1x DNA loading buffer (Orange G) were loaded into the wells. DNA was separated by electrophoresis under constant voltage conditions between 100-120V. Sizes of DNA fragments were estimated by comparison with fragments derived from 1kb or 100bp DNA ladders run in parallel. Resolved DNA fragments were visualized by placing agarose gel on a UV transilluminator at a wavelength of 254nm.

2.1.2 Denaturing polyacrylamide/urea gel electrophoresis

Samples from ribonuclease protection assays (RPA) were resolved through denaturing polyacrylamide gels. Prior to use, glass plates were carefully washed with detergent, rinsed with water and cleaned with 100% ethanol and left allowed to air dry. Plates were assembled according to the manufacturer's instructions using 0.8mm spacers. 6% Polyacrylamide/7M urea gels were made up by

dissolving 10.5g of urea in 3.75ml 40% acrylamide:bis-acrylamide (19:1), 2.5ml 10x TBE buffer made up to 25ml with nuclease-free water. 150ul ammonium persulphate (10%) and 25ul TEMED were added to the gel solution to accelerate polymerization and the gel solution was poured immediately. Wells were flushed well prior to sample loading which were run separated at constant voltage 250V for 3 hours.

Following electrophoresis gels were laid onto 3M Whatman paper and vacuum-dried on a BioRad Model 583 Gel drier at 80°C for 1 hour. Dried gels were exposed to BioMax MS autoradiographic film for 3-7 days at -80°C or to phosphorimager screens at room temperature for 3-7 days. Exon 1 specific MR fragments were quantified using a Fujifilm FLA-2000 phosphorimager and Aida v2.0 software.

2.2 PCR-based techniques

2.2.1 Polymerase Chain Reaction

PCR was carried out to generate DNA fragments containing promoter regions of MRβ and MRγ variants from rat genomic DNA. For each reaction, 0.1ug of rat genomic DNA was mixed with forward and reverse primers (final concentration 0.3uM each), 2.5 units of accuprime Pfx polymerase, 1x acumprime Pfx buffer containing 1mM $MgCl_2$ and dNTP (0.3mM each), made up to a volume of 50ul with amplification-grade water. A negative control containing water instead of DNA template was also carried out in parallel. All PCR reactions were performed in a thermal cycler using 0.2ml thin-walled eppendorf tubes. The conditions for PCR reactions are described in detail in Tab 2-1. PCR products were kept at 4°C and analyzed by electrophoresis on 0.8% agarose gels to determine the size of

amplification products.

2.2.2 Reverse transcriptase polymerase chain reaction

The DNA templates for synthesizing specific cRNA riboprobes for RPA (Chapter 4) and *in situ* hybridization (Chapter 5) were generated by reverse transcription-PCR (RT-PCR).

First strand cDNA was synthesized by reverse transcription from total RNA of rat hippocampus (for RPA) or cortical culture (for *in situ* hybridization). RNA (2ug) was denatured at 72°C for 10 minutes then added to the reverse transcription reaction which contained 5mM $MgCl_2$, 1x reverse transcription buffer, 1mM dNTP, 40 units RNase inhibitor, random primer (0.5ug) and 15 units AMV reverse transcriptase in a total volume of 20ul. The reaction mixture was incubated at room temperature for 10 minutes and then heated to 42°C for 2 hours to allow efficient reverse transcription from the primers and then to 95°C for 5 minutes to inactivate reverse transcriptase.

Synthesized cDNA (2ul) was then used for PCR amplification in a reaction mix containing 1x Taq polymerase buffer, 1.5mM Mg^{2+}, 0.2mM dNTP, forward and reverse primers (0.2uM of each primer) and 1.25 units Taq DNA polymerase. The PCR conditions are stated in Tab 2-2 and Tab 2-3. Amplified PCR products were analyzed by electrophoresis through 0.8% agarose gels.

Table 2-1 PCR conditions for generation of MRβ and MRγ promoter fragments

	Sample Size	Denaturation	Cycles	Thermal cycle conditions	Extension
pMRβ	300bp	95°C 2min	30	95°C, 15s; 59°C, 30s; 68°C, 2min	68°C, 10min
pMRγ	1.7kb	95°C 2min	30	95°C, 15s; 59°C, 30s; 68°C, 2min	68°C, 10min

Table 2-2. PCR conditions for generation of MR specific exon 1 DNA fragments (RNase protection assay)

	Sample Size	Denaturation	Cycles	Thermal cycle conditions	Extension
MRα	390bp	94°C 5min	35	94°C, 40s; 54°C, 40s; 72°C, 2min	72°C, 10min
MRβ	206bp	94°C 5min	35	94°C, 40s; 54°C, 40s; 72°C, 2min	72°C, 10min
MRγ	300bp	94°C 5min	35	94°C, 40s; 54°C, 40s; 72°C, 2min	72°C, 10min

Table 2-3. PCR conditions for generation of MR specific exon 1 DNA fragments (*In situ* hybridization)

	Sample Size	Denaturation	Cycles	Thermal cycle conditions	Extension
MRα	200bp	94°C 5min	35	94°C, 1min; 52°C, 30s; 72°C, 1min	72°C, 10min
MRβ	383bp	94°C 5min	35	94°C, 1min; 52°C, 30s; 72°C, 1min	72°C, 10min
MRγ	121bp	94°C 5min	35	94°C, 1min; 52°C, 30s; 72°C 1min	72°C, 10min

Table 2-4. Primers for promoter constructs

	Forward	Reverse
pMRβ	5'-TGCACTGGAGTTCCGTTTCC-3'	5'-CGGCGATAGAGAGTGGTTAG-3'
pMRγ	5'-ACCTTGAGCCCTGTGTTTGG-3'	5'-GAAGACCAAGAGCGGACTAG-3'

Table 2-5. Primers for generation of MR specific exon 1 DNA fragments (RNase protection assay)

	Forward	Reverse
MRα	5'-AGGAAGAGAGCCAACTTCAGGCTG-3'	5'-GTACTGTTGTTCGGAATAGCACCG-3'
MRβ	5'-AGCCTCCCTAACATGTCCTAG-3'	5'-GTACTGTTGTTCGGAATAGCACCG-3'
MRγ	5'-TCCTAGTCCGCTCTTGGTCTTC-3'	5'-GTACTGTTGTTCGGAATAGCACCG-3'

Table 2-6. Primers for generation of MR specific exon 1 DNA fragments (*In situ* hybridization)

	Forward	Reverse
MRα	5'-CGGGAGAAGAGAGCCAACTT-3'	5'-GTCGTCCTCTCGCCGTCTAC-3'
MRβ	5'-GGGTCTTACCGCTCGACTG-3'	5'-AGTCGCTGCACTCACCTTTT-3'
MRγ	5'-TTCCCTCCTAGTCCGCTCTT-3'	5'-ACGCACCCTGTATGCTTCTC-3'

2.2.3 Materials

Table 2-7 Materials for PCR based techniques and electrophoresis

PCR and RT-PCR	
Reverse transcription system	Promega
TaqBead polymerase kit	Promega
Pfx polymerase kit	Invitrogen
100mM dNTPs	Promega
Nuclease-free water	Promega
DNA purification kit	Roche pharmaceuticals
Agarose gel eletrophoresis	
Ethidium bromide	Sigma-Aldrich
Agarose	Lonza
Low-melting agarose	Invitrogen
1kb DNA ladder	Invitrogen
100kb DNA ladder	Invitrogen
Low mass DNA ladder	Invitrogen

2.3 Cloning of DNA

2.3.1 Blunt-ending of PCR products

PCR products for promoter constructs were blunt-ended by treatment with Klenow polymerase (5 units) in 1x DNA polymerase buffer, dNTP (100uM each), 0.5ul BSA in 20ul mixture for 10 minutes at room temperature. Reactions were terminated by incubation at 75°C for 10minutes.

2.3.2 Preparation of plasmid vector for cloning

The pGL3 vector was prepared for ligation with promoter fragments firstly by linearization with SmaI. For this, 2ug of empty vector was mixed with 1x restriction digest buffer, 10 units of restriction enzyme SmaI, made up to a volume of 20ul with distilled H_2O and incubated at room temperature for 2 hours. Gel electrophoresis was performed to confirm successful digestion. Linearized vector then underwent 5' dephosphorylation using calf intestinal alkaline phosphatase (10 units, CIAP) in 1x CIAP buffer for 30 minutes at 37°C. To terminate the reaction, CIAP was inactivated by addition of 2ul 0.5M EDTA (pH8.0) and incubation at 65°C for 20 minutes.

Riboprobe template construct was generated by TA cloning using pGEM T-Easy vector which was pre-linearized with EcoRV.

2.3.3 DNA purification

Vector DNA and PCR fragments were subjected to either gel purification or

normal purification. 30ul PCR products and linearized vectors were resolved through 1% gel made with low-melting point agarose stained with ethidium bromide (final concentration 0.2ug/ml). DNA was visualized using a UV transilluminator at a wavelength of 365nm and the required fragment was excised. DNA was released from the agarose using the Roche DNA purification kit according to the manufacturer's instructions. Briefly, excised pieces of gel containing DNA were dissolved in extraction reagent and incubated at 55°C for 10 minutes. DNA was isolated and purified by addition of isopropanol then passage through a filter spin column at 13,000rpm for 1 minute. Filter columns were washed twice with buffer containing 70% ethonal to remove residual salts before the DNA was eluted with 20ul elution buffer. The concentrations of recovered PCR products and vector plasmid were estimated by comparison with DNA bands from a low-mass DNA ladder on agarose gels.

2.3.4 Ligation of vector and PCR products

PCR inserts were ligated into plasmid vectors was using a ratio of 1:3 of vector and insert in a reaction containing 1x T4 DNA ligase buffer and 3 units of T4 ligase, made up to a total volume of 20ul with milli-Q water. Ligation reactions were carried out at 12°C for a minimum of 16 hours.

2.3.5 Transformation of DNA into bacteria

Ligation products were transformed into DH5α maximum efficiency competent cells. Cells were thawed on wet ice and 200ul aliquots transferred into chilled tubes. 1-1.5ul ligation mixture was added to the competent cells with gently swirling, followed by incubation on ice for 30 minute. Cells were then subjected to heat shock at 42°C for exactly 45 seconds and returned immediately onto ice

for a further 2 minutes. 1ml of SOC medium was added to the cells and incubated in an orbital shaking incubator at 180rpm at 37°C for 1 hour. Cells were spun at 3000 rpm for 30 seconds to allow sedimentation, 800ul medium was removed and cells were resuspended in the remaining 200ul SOC medium. The transformation mixture was plated out onto LB agar plates containing 100ug/ml ampicillin. Plates were allowed to air dry for 5-10 minutes, inverted and incubated at 37°C overnight.

2.3.6 Mini-preparation (small-scale preparation of plasmids DNA)

Positive recombinants were screened by small-scale preparation of plasmid DNA followed by restriction digestion. For each plate, 20 individual positive colonies and cultured in 2 ml LB culture medium containing 100ug/ml ampicillin in an orbital shaking incubator at 180rpm at 37°C overnight. Grown cultures were transferred into microcentrifuge tubes, and cells were sedimented by centrifugation at 6,000rpm for 3 minutes. Cell pellets were resuspended in 100ul ice-cold GTE buffer and incubated at room temperature for 5 minutes. Cells were then mixed with 200ul freshly-prepared 0.2M NaOH and 1% SDS and incubated on ice for 5 minutes before the addition of 150ul 5M potassium acetate (pH4.8) and incubation on ice for a further 5 minutes. Lysed cells were centrifuged at 13,000rpm at 4°C for 5minutes and the resulting supernatant containing plasmid DNA was collected and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by extraction with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 2 volumes 100% ethanol, collected by centrifugation, washed with 70% ethanol and air-dried. Each DNA sample was dissolved in 20ul nuclease-free water. Residual RNA was removed by treatment with 20units of RNase A at 37°C for 20 minutes. The concentration of plasmid DNA was measured by UV spectrophotometry.

2.3.7 Large-scale (maxi) preparation of plasmid DNA

Positive recombinants containing insert in the correct orientation as determined from the initial screening process were cultured for large scale plasmid DNA preparation. Initially, a sample from that colony was seeded in 2ml LB medium containing 100ug/ml ampicillin at 37°C for 6 hours. The culture was then transferred to 500ml ampicillin-containing LB medium and incubated in an orbital shaking incubator at 37°C for a further 16 hours.

Cells were sedimented by centrifugation in pre-chilled tubes at 6,000 rpm for 5 minutes at 4°C in a Beckman J2-MC centrifuge. Cell pellets were resuspended in 12ml ice-cold GTE and mixed with 24ml freshly prepared 0.2M NaOH and 1% SDS and allowed to incubate on ice for 10 minutes. Cells were then mixed with 16ml ice-cold 5M potassium acetate placed on ice for a further 10 minutes. Cell lysates were centrifuged at 6,000rpm for 10 minutes at 4°C and collected supernatant was passed through gauze filters to remove residual cell debris. Plasmid DNA was retrieved by precipitation with 32ml isopropanol for 30 minutes at room temperature, and centrifugation at 10K rpm for 3 minutes. Air –dried DNA pellets were redissolved in 2.2ml TE buffer and subjected to caesium chloride ($CsCl_2$, 1g/ml) gradient ultracentrifugation at 70K rpm for 20 hours in the presence of 0.4mg/ml ethidium bromide. Plasmid DNA contained within the ethidium bromide layer was collected and subjected to further ultracentrifugation at 100,000rpm for 4 hour, re-collected then extracted from the ethidium bromide with chloroform. Finally, plasmid DNA was purified by dialysis in TE buffer for a minimum of 18 hour with at least 3 changes of dialysis buffer.

The concentration of plasmid DNA was measured by UV spectrophotometry.

2.3.8 DNA sequencing

Plasmid DNA containing the inserts of interested were verified by DNA sequencing using appropriate sequencing primers via a commercial company (The Sequencing Service, University of Dundee)..

2.3.9 DNA concentration

Plasmid DNA concentrations were assessed by measurement of optical density using a GeneQuant RNA/DNA spectrophotometer. Diluted DNA (total 100ul) was transferred into quartz cuvettes and absorbance values measured at a wavelength 260nm. To determine the purity of the DNA, absorbance values were also measured at 280nm and the ratio of absorbance at 260/280nm calculated. Values between 1.7 and 1.9 indicated purity of 80% to 100%. Each sample was measured in duplicate and mean values taken to be the concentration of that sample.

2.3.10 Restriction digests

To determine the orientation of insert DNA in the plasmid, restriction mapping was performed. Each reaction was comprised of 1ug plasmid DNA, 2ul 10x appropriate digestion buffer, 0.2ug BSA and 10 units of restriction enzyme(s), in a total volume of 20ul. Restriction digests were incubated at 37°C for 1-2 hours, then fragments were visualized by gel electrophoresis. The enzyme(s) used for promoter construct pMRβ 300 were KpnI and SacII, for pMRγ was XhoI. The enzyme for all riboprobe plasmid template constructs was NotI.

2.3.11 Materials

Table 2-8 Materials for DNA cloning

pGL-3 basic	Promega
pGEM-T-easy vector system	Promega
DH5α competent cells	Invitrogen
Ampicillin	Sigma-Aldrich
LB agar	Sigma-Aldrich
CIAP (including buffer)	Roche
SOC medium	Invitrogen
Phenol/chloroform/isoamyl alcohol	Sigma-Aldrich
Chloroform/isoamyl alcohol	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
All restriction enzymes	Promega

2.4 Cell culture

2.4.1 Poly-D-lysine preparation and plate coating

Poly-D-lysine was dissolved in autoclaved tissue culture grade H_2O to give a final concentration of 50ug/ml and a sufficient volume was used to coat 6-well plates for 1-6 hours at 37°C. Once coated, poly-D-lysine was aspirated from the wells and plates were left to dry in a standard tissue culture incubator overnight. Dried poly-D-lysine coated plates were stored then at 4°C. Prior to use, wells were briefly rinsed with 1x PBS.

2.4.2 Preparation of rat primary cortical cultures

Rat primary cortical cultures were prepared from the foetuses of E18 ± 0.5 day old Wistar rats. Dams were sacrificed by cervical dislocation and foetuses were quickly removed from the uterus. Cerebral cortices were dissected from each foetal brain and the meninges were carefully removed and discarded. Cortices were finely chopped then digested in Hanks balanced Salt solution (HBSS) containing trypsin (1800U/ml) and DNase I (500U/ml) for 10 minutes at 37°C with occasionally agitation. Trypsinization was stopped by addition of trypsin inhibitor solution (0.45mg/ml) and digested cells were collected by centrifugation at 1000rpm for 5 minutes at room temperature. Cells were resuspended in 10ml DNase I solution (500u/ml) and dissociated by trituration through a 21-gauge needle. Cells were centrifuged at 1000rpm for 10 minutes, resuspended in Neurobasal A culture medium containing B-27 supplement, 1% L-glutamine (0.5mM) and 1% penicillin/streptomycin (50U/ml) and plated at a density of 2×10^6/well (6 well plates for culture), 8×10^5/well (12-well plates for immunohistochemistry) or 1.5×10^5/well (48-well plates for time-response curve).

Cultures were incubated under standard tissue culture conditions at 37°C for 14 days with culture medium replaced on day 7.

2.4.3 Preparation of collagen

Collagen type IV was dissolved in 0.1M glacial acetic acid solution, mixed at room temperature for 2 hours and then sterilized by incubation with 10ml of chloroform which was carefully added to form a layer beneath the collagen. After overnight incubation at 4°C, the aqueous upper layer containing the collagen was aseptically removed and stored in a sterile vial at 4°C until ready for use.

2.4.4 Treatment of cultureware with collagen

Sufficient collagen was added to cover the surface of $75cm^3$ standard tissue culture flasks or the bottom of multiwell plates and incubated overnight in a standard tissue culture incubator. The collagen was then removed and coated flasks and plates were left to dry at 37°C in tissue culture incubators. Prior to use, the flasks/plates were rinsed twice with 1x PBS.

2.4.5 Maintenance of PC12 cell line

Rat pheochromocytoma PC12 cells were maintained as monolayers in 10ml complete medium comprised of Delbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% horse serum (HS), 1% L-glutamate(2mM) and 1% penicillin/streptomycin (50U/ml) in collagen-coated flasks (prepared as described above). Cells were routinely incubated in standard tissue culture incubators at 37°C under humidified conditions of 95% atmospheric

air/5% CO_2. Confluent cells were split every 4-5 days: monolayers were initially rinsed with 2ml trypsin/EDTA then detached from flasks by incubation at 37°C with 5ml trypsin/EDTA. To split cells, 4.5ml of the cell suspension was removed and the remainder was diluted with complete medium and grown as described.

2.4.6 Cell harvest

PC12 cells were seeded into collagen coated 6-well plates for experimental set-ups as follows: monolayers were detached from flasks as described above and transferred to falcon tubes containing 10ml complete medium. Cells were collected by centrifugation at 1000rpm at room temperature for 5 minutes. The supernatant was carefully aspirated and the cell pellet was resuspended in 10ml complete medium.

The number of harvested cells was counted with the aid of an improved Neubauer haemocytometer. The original cell suspension was then made up to the given concentration of cells with the required volume of complete medium. For experiments involving undifferentiated cells, cells were seeded at a density of $1\times10^4/cm^2$ and for experiments involving differentiated cells, cells were seeded at a density of $6\times10^3/cm^2$.

2.4.7 Preparation of NGF

PC12 cells were differentiated using nerve growth factor (NGF). Lyophilised NGF was prepared by dissolution in glucose-free DMEM culture medium to give a stock concentration of 0.1mg/ml and stored in 10ul aliquots at -80°C until ready for use.

2.4.8 Establishment of differentiated PC12 cells

Undifferentiated PC12 cells in complete medium were seeded into 6 or 12 well culture plates and left to grow for 24 hours. Culture medium was aspirated and replaced with serum-free DMEM medium containing 2mM L-glutamine and 50U/ml penicillin/streptomycin and returned to the tissue culture incubator for at least 1 hour. NGF was added to each well at a concentration of 100ng/ml and allowed to grow for a further 3 days before experimentation.

To ensure PC12 cells exposed to NGF were terminally differentiated, 3 different assessments were made:

i) Cell morphology

Cell morphology was observed at various timepoints using an optical light microscope and images were recorded using an MCID M4 image analysis system.

ii) Cell proliferation

Cell proliferation was determined using the MTS proliferation system. MTS assay buffer was brought to a temperature of 37°C and added to each well to give a dilution of 1:5. For negative controls, wells containing serum-free DMEM medium in the absence of cells were used. Cells were returned to tissue culture incubators for 2 hours then 50ul culture medium was removed from each well and the amount of formazan product present in the medium was measured in a 96-well plate reader at a wavelength of 490nm. Samples from each well were measured in duplicate.

iii) Expression of tyrosine hydroxylase

PC12 cells previously exposed to NGF for 3 days were subjected to immunocytochemistry to detect tyrosine hydroxylase and compared with undifferentiated PC12 cells. Briefly, after removal of culture medium, cells were

washed twice with 1x PBS then fixed with 4% ice–cold paraformaldehyde for 30 minutes at 4°C. Fixed cells were washed repeatedly with 1x PBS, incubated with 0.6% hydrogen peroxide for 15 minutes to quench endogenous peroxidase, then permeabilized with 0.3% Triton X100 in 1x PBS for 3 minutes. To prevent non-specific binding cells were incubated with 1.5% horse serum for 1 hour at room temperature before overnight incubation at 4°C with an anti-tyrosine hydroxylase monoclonal antibody (1:500 dilution). Cells were washed three times with 1x PBS and incubated with a biotinylated anti-mouse secondary antibody (1:5000 dilution) for 1 hour at room temperature. Bound antibody was detected using the Vectastain *Elite* ABC kit according to the manufacturer's instructions and visualized colorimetrically with diaminobenzidine (DAB) Differentiated cells without primary antibody incubation served as the negative control. Immuno-fixed cells were examined under a light microscope and images taken using MCID image analysis system.

Immunocytochemical analysis of primary cortical culture

To determine the percentage population of neurons versus glia in rat primary cortical cultures, immunocytochemistry was used to detect the neuronal marker microtubule-associated protein 2 (MAP2) and the glial marker, glial fibrially acidic protein (GFAP). Cells were fixed, incubated in a rabbit polyclonal anti-GFAP (1:500) or a monoclonal anti-MAP2 (1:200) and processed for detection as described above with the appropriate biotinylated secondary antibody. Immunostaining was observed by light microscopy and the proportion of neuron to glial cells was calculated by counting the number of stained neurons and glia from 10 randomly selected fields per well.

2.4.9 Cell stressors

Undifferentiated and differentiated PC12 cells were exposed to six different types

of cellular stressors: staurosporine, NMDA, H_2O_2, hypothermia, OGD and combined OGD and hypothermia (OGD&hypothermia) for given periods of time.

Staurosporine was dissolved in anhydrous dimethylsulphoxide (DMSO) to a stock concentration of 10mM, and stored in 5ul aliquots at -80°C. Aliquots were thawed and subjected to serial dilution in serum-free DMEM culture medium before being added to cells to give final concentrations ranging from 0.1nM to 10uM.

NMDA was dissolved in DMEM to give a stock concentration of 1M and pH adjustments were made were necessary. Serial dilutions were made from neutralized stock NMDA and added to the cells to give final concentrations ranging from 10uM to 10mM.

Cells were subjected to hypothermia by incubation at 33°C in a standard tissue culture incubator. Normal culture medium was aspirated and replaced with medium pre-warmed to 33°C. Cells were incubated at the 33°C for a given time then returned to 37°C for a total duration of 24 hours.

Oxygen-glucose deprivation (OGD) was performed in a custom made temperature- and CO_2-controlled chamber containing $1\%O_2/5\%CO_2/94\%N_2$ (200bar). Cells were introduced into the glove box where the normal culture medium was aspirated and replaced with glucose-free DMEM culture medium previously pre-equilibrated in the glove box for at least 30 minutes. Cells were incubated in the hypoxic chamber for a given time and returned to standard culture conditions with the addition of 0.45% glucose (wt/vol).

Cells were exposed to OGD&hypothermia as described for OGD with temperature set at 33°C.

2.4.10 LDH assay

The amount of lactate dehydrogenase (LDH) released by cells was measured to determine the extent of cell damage following exposure to cell stress using a commercially available kit. Briefly, 50ul culture medium from each well was transferred to a 96-well plate, mixed with 50ul LDH assay buffer and incubated at room temperature for 30 minutes in the dark. The reaction was stopped by addition of acetic acid (1M) and absorbance was measured at 490nm. To measure total LDH, cells were lysed with 1% Triton X100 at 37°C for 45 minutes then 50ul cell lysate was analysed for LDH as described above.

To calculate the amount of LDH released by cells in response to cell stress, the percentage of cell damage was calculated using the equation described in Appendix 2.

2.4.11 Transient transfection

Undifferentiated PC12 cells were seeded at the required density in collagen coated multiwell plates and incubated under standard culture conditions. Cells were transiently transfected with relevant reporter plasmids when they had reached 50-60% confluency. Initially, culture medium was aspirated from cells and replaced with antibiotic-free complete DMEM medium at least 1 hour prior to transfection.

For transfection studies in undifferentiated PC12 cells, 0.5ug promoter insert-containing plasmid DNA per well was mixed with 0.5ug of the β-galactosidase encoding pCH110 plasmid (internal control) in 50ul Optimem™

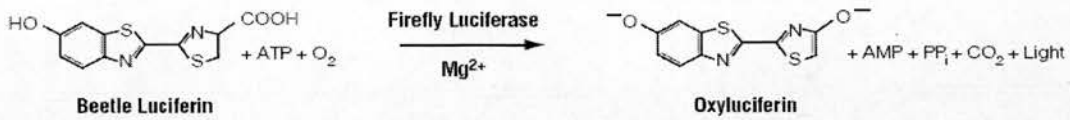
medium. The DNA mixture was transfected into cells using lipofectamine 2000™ (1ul/ug DNA) according to the manufacturer's instructions. Cells transfected with 0.5ug promoterless pGL3-basic vector served as the baseline control. Transfected cells were subjected to cell stress 24 hours later.

For promoter studies involving differentiated PC12 cells, initially 0.375ug insert containing plasmid and 0.375ug pCH110 plasmid were transfected into 50-60% confluent undifferentiated cells as described above. Transfected cells were differentiated 24 hours later: culture medium was replaced with serum-free antibiotic containing DMEM medium then cells were treated with NGF (100ng/ml) and maintained under standard incubation conditions for a further 3 days before exposure to cell stress (as described in 2.4.9).

2.4.12 Luciferase and β-galactosidase assays

24 hours after the start of cell stress, cells were harvested for measurement of luciferase and β-galactosidase (β-gal) activity. Cells were washed with 1x PBS and lysed in luciferase lysis buffer. Cell lysates were collected and centrifuged at 13,000rpm for 2 minutes at room temperature to remove debris. To measure luciferase activity, 40ul of cell lysate from each sample was mixed with 100ul 2x luciferase assay buffer and 5ul 100mM ATP, and placed into a single–tube luminometer then 100ul 1mM beetle luciferin was injected to the lysate mixture. Luciferase activity for each sample was performed in triplicate. The amount of chemiluminescence emitted was directed related to luciferase activity as described in the equation below:

A



B

β-galactosidase

Nitrophenyl-β-galactoside (ONPG) ⟶ galactose + ortho-nitrophenol + yellow light

Fig 2-1. Bioluminescent reaction catalyzed by firefly luciferase. (Promega) (A) and β-galactosidase (B)

β–gal activity was measured according to the method developed by Jain and Magrath (Jain *et al.* 1991), using the Tropix/Galacto Light kit. For each sample (performed in triplicate), 10ul cell lysate was mixed with 67ul Galacto-Light assay buffer and incubated at room temperature for a minimum of 20 minutes at room temperature. β–gal activity was measured in a single-tube luminometer following addition of 100ul chemiluminescent accelerator solution. Luciferase activity was then expressed relative to β-gal activity.

2.4.13 Materials

Table 2-9 Materials for cell culture, stressors, transfection and reporter assay

General cell culture	
6-well cell culture plate 12-well cell culture plate 48-well cell culture plate 96-well cell culture plate 60mm culture dishes 75mm^2 cell culture flasks 5 ml sterile pipettes 10 ml sterile pipettes 25 ml sterile pipettes	Corning
Plate/dish coating	
Poly-D-lysine	Sigma-Aldrich
Collagen (type IV)	Sigma-Aldrich
PC12 cell culture	
DMEM medium	Lonza
DMEM medium (glucose-free)	Invitrogen
HBSS	Lonza
Fetal bovine serum	Lonza
Horse serum	Invitrogen
L-glutamate (200mM)	Lonza
Penicillin/streptomycin (5000U/ml)	Lonza
trypsin/EDTA (0.25% trypsin; 1mM EDTA)	Lonza
NGF	Upstate
Glucose solution (45%)	Sigma-Aldrich
Primary cortical culture	
Neurobasal medium	Invitrogen
B27 supplement	Invitrogen
Trypsin (powder)	Sigma-Aldrich
Trypsin inhibitor	Sigma-Aldrich
DNase	Sigma-Aldrich
Establishment of differentiated PC12 cells	
MTS assay kit	Promega
LDH assay kit	Promega
Mouse anti-TH monoclonal antibody	Chemicon
Mouse anti-MAP2 monoclonal antibody	Sigma-Aldrich
Rabbit anti-GFAP polyclonal antibody	Sigma-Aldrich
Vectastain elite ABC kit	Vector Laboratories
DAB	Vector Laboratories

Cellular stressors	
NMDA	Tocris
Staurosporine	Sigma-Aldrich
H_2O_2	Sigma-Aldrich
Transfection and reporter gene assay	
Optimem medium	Invitrogen
Lipofectamine 2000 transfection reagent	Invitrogen
Firefly Bettle Luciferin	Promega
Galacto light plus assay kit	Applied biosystems
Coenzyme A (10mg)	Sigma-Aldrich
Tricine	Sigma-Aldrich

2.5 RNA Analysis

All RNA work was performed with utmost care to avoid RNA degradation by ribonucleases. All materials were autoclaved and equipment was treated with RNAzap™. All solutions were made up with diethypyrocarbonate (DEPC)-treated H_2O.

2.5.1 RNA extraction

Total RNA was isolated from primary cortical cultures as follows: cells were washed with 1x PBS, lysed with Trizol reagent (1ml per $10cm^2$) and allowed to incubate at room temperature for a minimum of 5 minutes to permit the complete dissociation of nucleoprotein complexes. Chloroform was added to each sample, mixed vigorously by hand then incubated at room temperature for a further 2-3 minutes before centrifugation at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase containing RNA was transferred carefully into fresh tubes, and RNA was precipitated with 500ul isopropyl-alcohol for 10 minutes. RNA pellets were retrieved by centrifugation at 12,000rpm at 4°C for 10 minutes, washed with 75% ethanol, air-dried and resuspended in 30ul nuclease-free water. RNA samples were incubated at 55°C for 10 minutes to thoroughly dissolve RNA.

The concentration and purity of RNA was assessed by measurement of optical density as described in 2.1.1.11. Purity ratios ranged from 1.6 to 1.8 (75% to 100%). The integrity of RNA was also assessed by agarose gel eletrophoresis to verify intact 28S and 18S ribosomal RNA.

2.5.2 Synthesis and purification of riboprobes

Sense and antisense riboprobes were generated by *in vitro* transcription of linearized insert-containing pGEM T-easy plasmids in reactions containing 1x transcription buffer, 10mM DTT, 40 units recombinant RNase inhibitor, ATP, CTP and UTP mixture (0.5mM each), GTP (10uM), 0.2-0.5ug linearized template DNA, [α-^{32}P]-GTP (3000Ci/mmol) and 20units of SP_6 or T_7 RNA polymerase. Reactions driven by SP_6 polymerase were incubated at 40°C whereas those using T_7 polymerase were incubated at 37°C for 2 hours. Reactions were terminated by the addition of 5 units RNase-free DNase and incubated for 15 minutes at 37°C.

Radiolabelled riboprobes for *in situ* hybridization were synthesized as above except that [α-^{35}S]-UTP was used.

2.5.3 Purification of riboprobe

Radiolabelled riboprobes were purified using DNA Nick™ columns according to the manufacturer's instructions. Reaction mixtures were loaded onto pre-equilibrated columns and allowed to penetrate into the columns by addition of nuclease-free water (400ul). Purified samples were then eluted with further 200ul nuclease-free water. The specific activity of each purified riboprobe was counted using a Wallac 1450 Microbeta Plus liquid scintillation counter. The integrity of each riboprobe was validated by resolution through 6% polyacrylamide gel electrophoresis with 10^5cpm of riboprobe loaded

2.5.4 RNase Protection Assay (RPA)

RPAs were performed using the commercially available HybSpeed™ RPA kit.

Total RNA (25ug) and carrier yeast tRNA (25ug) were precipitated in 0.1M ammonium acetate, 2.5 volume of 100% EtOH and 2.5×10^5cpm MR probe (MRα, MRβ or MRγ) and 1×10^4cpm β-actin probe (internal control) at -20°C for a minimum of 20 minutes. Precipitated RNA was collected by centrifugation at 13,000rpm for 15 minutes and resuspended in 10ul hybridization buffer. Samples were denatured at 95°C and subjected to intermittent vortexing until each pellet had completely dissolved. Samples were hybridized at 68°C for 10 minutes to 1 hour then treated with 0.5unit RNase A and 20units RNase T1 at 37°C for 30 minutes with occasional agitation, followed by addition of 1.5 volumes of inactivation/precipitation buffer (150ul) and 75ul ethanol and incubation at -20°C for least 1 hour. Protected double-stranded RNA pellets were obtained by centrifugation at 13,000rpm for 15 minutes at room temperature and resuspended in 6ul polyacrylamide gel loading buffer. Samples were denatured at 95°C for 3 minutes, chilled on ice and loaded onto the gels as described in 2.1.2.

2.5.5 *In situ* hybridization

Cryostat coronal sections of frozen brain (12um) were cut and thaw-mounted onto Superfrost™ microscope slides. Mounted sections were briefly air dried and stored under desiccated conditions at -80°C until ready for use.

Fixation

Sections were allowed to warm to room temperature then fixed in ice-cold 4% paraformaldehyde for 10 minutes. Slides were rinsed twice in 1x PBS and incubated in 0.1M triethanolamine for 10 minutes, washed again in 1x PBS, then dehydrated through an ascending ethanol series (70%, 80% and 95%) and air-dried.

Prehybridization and hybridization:

Fixed sections were treated with 1x prehybridization buffer, coverslipped and incubated under humid conditions at 50°C for a minimum of 3 hours. Hybridization mix comprised of 10^7 c.p.m. radiolabelled probe in 1x hybridization buffer and deionized formamide (12.5mM) was denatured at 75°C then chilled on ice before DTT was added (10mM). After prehybridization buffer was replaced with hybridization mixture, slides were re-coverslipped and returned to the hybridization oven for a further 16-18 hours.

Following hybridization, sections were washed three times in 2x SSC then treated with RNase (0.09ug/ml) in RNase buffer at 37°C for 1 hour. Slides were washed once in 2x SSC at room temperature then subjected to two stringent washes in 0.1x SSC at 60°C for 1 hour. Finally brain sections were dehydrated through an ascending series of ethanol (50%, 70% and 90%) containing ammonium acetate (0.3M), air dried then exposed to Kodak MR autoradiographic film for 2-3 weeks.

NTB2 photoemulsion and development of the slides:

Slides were exposed to photographic liquid emulsion to enable visualization of the hybridization signal in the form of silver grains. All procedures were carried out in the dark room under a safety light. Photographic emulsion was melted in a water bath at 42°C and diluted with DEPC H_2O (1:1 vol/vol). Slides were gently immersed in the liquid emulsion then allowed to dry overnight at room temperature. Dried slides were placed in light-tight boxes, wrapped in foil paper and exposed for 3 weeks at 4°C.

Slides were brought to room temperature then developed in Kodak 19 photographic developer solution at 15°C. Developed slides were fixed in 20% Kodak Fixative buffer for 5 minutes, rinsed with water then air-dried at room

temperature. Developed slides were counter-stained with 1% pyronin Y and mounted in DEPEX mountant.

For each hippocampal subfield, mRNA expression levels were quantified by measuring the mean total intensity (pixels) of silver grains per neuron per subregion by computer assisted quantitative grain counting using an MCID M4 image analysis system. For each animal, 3 adjacent sections were analyzed with 20 neurons measured randomly per subfield. The results were analysed and obtained by the investigator blinded to experimental condition.

2.5.6 Materials

Table 2-10 Materials for RNA analysis, RPA and *in situ* hybridization

Radioactive materials and riboprobe generation	
5'-[α-^{32}P]-rGTP 3000Ci/mmol	Amersham
5'-[α-^{35}S]-rUTP 3000Ci/mmol	Amersham
Liquid scintillant	Zinsser Analytic
Nick™ columns	Amersham
Riboprobe in vitro transcription system™	Promega
RNA extraction	
Trizol™	Invitrogen
RNAzap™	Ambion
Dithiothreitol (DTT)	Sigma-Aldrich
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich
RPA	
HybSpeed™ Ribonuclease protection assay kit	Ambion
β-actin antisense plasmid templates	Ambion
Whatman paper 3MM	Whatman
Autoradiograph film (Kodak Biomax MS)	Sigma-Aldrich
RNA marker template DNA	Ambion
TEMED	Sigma-Aldrich
Urea	Sigma-Aldrich
Ammonium persulphate	Sigma-Aldrich
Acrylamide:bis-acrylamide (19:1)	Sigma-Aldrich
Ultrapure water	Sigma-Aldrich
In situ hybridization	
Deionised Formamide	Sigma-Aldrich
Formamide	Sigma-Aldrich
Yeast tRNA	Sigma-Aldrich
20x SSC	Invitrogen
Paraformaldehyde	VWR
Na_2HPO_4	BDH laboratory
$N_aH_2PO_4$	BDH laboratory
Triethanolamine	BDH laboratory
KCl	BDH laboratory
KH_2PO_4	BDH laboratory

Ammonium acetate	Sigma-Aldrich
50x Denhardt's	Sigma-Aldrich
Sonicated salmon sperm DNA	Sigma-Aldrich
Dextran sulphate salt	Sigma-Aldrich
Kodak autoradiography emulsion	VWR
Pyronin Y	Anachem
DEPEX	Fluka
Superfrost plus micro slide	BDH laboratory
Autoradiograph film (Kodak Biomax MR)	Sigma-Aldrich

2.6 Data analysis

Blinded assessment was carried out for obtaining and analysing the results from reporter gene assays, RPA and *in situ* hybridization. All the data were represented as the mean ± S.E.M for the number of experiments indicated. Statistical analysis was performed by one-way ANOVA for the comparison in all experiments except that two-way ANOVA was carried out for *in situ* hybridization followed by *post-hoc* one-way ANOVA (chapter 5). Values of $p<0.05$ were considered statistically significant.

2.7 Equipments

Table 2-11 List of equipments

Temperature-controlled hypoxia chamber	Wolf laboratories Ltd
Nitrogen (oxygen free)	BOC gases
Hoeffer vertical gel system (for RPA)	Bright Instruments Ltd
Single-tube Luminometer	Berthold Technologies GmbH& Co.
Mode 583 gel drier	Bio-Rad Laboratories
Cryostat	Leica Microsystems
Fuji Film FLA-2000 phosphorimager	Sigma-Aldrich
Fuji BAS phosphorimager screen	Sigma-Aldrich
MCID M4 image analysis system	MCID Research Imaging, Canada
Hybridization oven	Weiss-Gallenkamp
Gene Quant RNA/DNA calculator	Pharmacia
Eppendorf bench top microfuge	Kendro
Wallac 1450 Microbeta Plus liquid scintillation counter	Perkin and Elmer
Thermal cycler	Techne Inc
Orbital shaking incubator	Weiss-Gallenkamp
Microbiology Incubator	Weiss-Gallenkamp
J2-MC centrifuge (mini- and maxi-prep)	Beckman Coulter
Horizontal Electrophoresis apparatus	Bio-Rad Laboratories
Optical light microscope	Zeiss
Optimal TLX ultracentrifuge	Beckman Coulter
UV transilluminator	Uvitec Ltd
Statistical analysis (Sigma Stat 3.5; Sigma plot 10)	SPSS Inc.

2.7 Solutions

Table 2-12 Solutions

5M NaCl	146g NaCl was dissolved in 400ml DEPC H_2O and the volume was leveled up to 500ml
5M K acetate	Dilute 60g potassium acetate, 11.5ml of glacia acetic acetate in 28.5ml H_2O
50x TAE	121g Tris, 28.5ml glacial acetic acid and 50ml 0.5M EDTA (pH8.0) were dissolved in 500ml dH_2O.
10x TBE	108g Tris, 55g Boric acid, 40ml of 0.5M EDTA were dissolved in 1L dH_2O
Tris-EDTA (TE)	10mM Tris-HCL (pH8.0), 0.1mM EDTA
DNA loading buffer	10 mM Tris-HCl (pH 7.5), 0.15% orange G, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA.
4% Paraformaldehyde	2.4g NaH_2PO_4 and 11.35g Na_2HPO_4 were dissolved in 800ml DEPC H_2O at 80°C. 40g paraformaldehyde was added to the solution until completely dissolved. The volume was adjusted to 1L and kept at 4°C prior to use.
0.5 M EDTA	800ml DEPC H_2O was added to 186.1g EDTA pH was adjusted to 8.0 with NaOH and the volume adjusted to 1L.
1M Tris-HCL (pH8.0) 1M Tris-HCL (pH7.5)	121.1g Tris was added to 800ml water. pH was adjusted to 8.0 or 7.5 with concentrated HCL and the volume to 1000ml with water
10x PBS	80g NaCl, 29g Na_2HPO_4, 2g KH_2PO_4 and 2g KCl were dissolved in 900ml DEPC H_2O first. The volume was adjusted to 1L and then autoclaved
0.1 Triethanolamine	13.3ml triethanolamine was added to 800ml DEPC H_2O. pH was adjusted to 8.0 with HCL and the volume adjusted to 1L.
1M DTT solution	0.3g DTT was dissolved in DEPC H_2O.
10M NaOH	40g NaOH was dissolved in 100ml dH_2O.
DEPC H_2O	dH_2O treated with 0.1% diethylpyrocarborate and left at room temperature for 20min to 1 hour before autoclaving.
2x pre-hybridization buffer	1.2M NaCl, 20mM Tris (pH7.5), 2x Denhardt's solution, 2mM EDTA, 1mg/ml salmon testis DNA and 0.2mg/ml Yeast tRNA in 10ml DEPC H_2O
2x hybridization buffer	0.2g/ml Dextran sulphate, 1.2M NaCl, 20mM Tris (pH7.5), 2x Denhardt's solution, 2mM EDTA,

	0.2mg/ml salmon testis DNA and 0.2mg/ml Yeast tRNA in 10ml DEPC H_2O.
GTE	50ml glucose, 25mM Tris-HCL (pH8.0), 10ml EDTA
10% SDS	10g SDS was added into 80ml dH_2O and heated to 60°C until completely dissolved. pH was adjusted to 7.5 and the volume was to 100ml with dH_2O.
1M $MgSO_4$	Dissolve 12g $MgSO_4$ in 80ml dH_2O and the volume was adjusted to 100ml.
0.1M $MgCO_3$	2.4g $MgCO_3$ was added to 50ml dH_2O. Vortex upon use
2x Luciferase assay buffer	0.359g Tricine, 0.514g DTT, 0.2M EDTA, 2mM $MgCO_3$, 5mM $MgSO_4$ and 10mg Coenzyme A were in 50ml dH_2O.
Luciferase lysis buffer	25mM Tris (pH8.0), 2mM DTT, 1% Triton X100, 10% glycerol
RPA gel loading buffer (provided by Ambion)	95% formamide/0.025% xylene, cyanol/0.025% bromophenol blue, 18 mM EDTA, 0.025% SDS

Chapter 3

PROMOTER ACTIVITY OF THE MINERALOCORTICOID RECEPTOR VARIANTS IN RESPONSE TO DIFFERENT CELLULAR STRESSORS

3.1 Introduction

For any given gene, transcription is primarily regulated by the activity of its promoter. It is now increasingly recognized that many genes have multiple promoters which generate distinct and uniquely regulated transcripts from a single gene (Landry *et al.* 2003). Previous studies have revealed that the rat MR gene gives rise to three MR mRNA variants (α, β, γ) each encoding alternative 5'-untranslated regions. Transcription of each mRNA variant appears to be under the control of its own promoter. Similarly, the human MR gene generates two mRNA variants under the control of two separate promoters (P1 and P2) (Zennaro *et al.* 1996). Each of the MR variants has been shown to be expressed in a tissue- and development-specific manner (Kwak *et al.* 1993; Vazquez *et al.* 1998; Pascual-Le Tallec *et al.* 2004). A fragment of the rat MRα promoter was successfully cloned and found to respond to corticosteroids in a neuronal cell line (Castren *et al.* 1993). However, the exact function of each exon1 variant or other factors which regulate MRα, MRβ or MRγ promoter activity have yet to be identified.

Since MR expression is increased in response to cell injury (Macleod *et al.* 2003) but how this is regulated at the transcript level is not known, this chapter focuses on how injury-induced MR is regulated at the promoter level. Experiments were

designed to specifically answer the following:

(i) If the MR promoters are differentially regulated by cellular stress.

(ii) If promoter activity is specific to certain types of cellular stressor.

(iii) If promoter activity in response to cellular stress is dependent on cell type.

3.2 Results

3.2.1 Optimisation of experimental conditions

The promoter activity of each rat MR variant was investigated using a luciferase reporter assay whereby each promoter fragment was placed upstream of the pGL-3 firefly luciferase reporter construct and co-transfected with pCH110 encoding β-galactosidase into PC12 cells. The PC12 clonal cell line is derived from a pheochromocytoma of the rat adrenal medulla (Greene *et al.* 1976). These cells were chosen because they undergo differentiation in response to nerve growth factor (NGF): they cease proliferation, extend neurites and express neuronal markers associated with sympathetic neurons (Greene *et al.* 1976). As such they have been used extensively as a model system for primary neuronal cells. Here, both undifferentiated and differentiated PC12 cells were used as models to compare the activity of the MR promoters in essentially two different cell types (non-neuronal and neuronal-like).

3.2.1.1 Establishment of conditions for PC12 cell differentiation

The conditions for differentiating PC12 cells were first established using three different methods to verify the transition of PC12 cells from an undifferentiated to differentiated state: (i) observation of morphological changes by light microscopy,

(ii) measurement of the rate of cell proliferation and (iii) immunohistochemical detection of the sympathetic neuronal differentiation marker tyrosine hydroxylase.

Morphological changes:

Undifferentiated PC12 cells were cultured in serum-free DMEM with 100ng/ml NGF for 6 days, with NGF replenished on the 3rd day. As shown in Fig 3-1, undifferentiated PC12 cells underwent dramatic morphological changes during the process of differentiation. Undifferentiated PC12 cells display a round or elliptical shape (Fig 3-1 A) with no extensions from the cell body. Exposure to NGF caused cells to develop neurite-like projections within 24 hours. After 3 days of exposure to NGF, these projections extended considerably to form connections with other cells and eventually gave rise to an apparent neuronal network. This phenotype remained stable throughout the 7-day experimental time frame. Based on these microscopic observations, this suggests that undifferentiated cells accomplish differentiation by 3 days.

Cell proliferation

Since PC12 cells cease to proliferate when they undergo NGF-induced differentiation, measuring the rate of proliferation provides a useful indication of the differentiation status of these cells. In this experiment, the rate of cell proliferation was measured using the MTS assay which is based on the ability of mitochondria to metabolically convert the compound MTS [3-(4,5-dimethylthiazol -2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] into the formazan product which is then quantified colorimetrically. The intensity of the converted formazan is directly proportional to the number of living cells. As Fig 3-2 shows, the rate of production of the converted formazan in PC12 cells either in the presence or absence of NGF was similar over the first 3 days, indicating there

was no significant difference in cell proliferation. However by day 4, the amount of converted formazan in undifferentiated cells was significant higher (36%±1.5%; $p<0.001$) compared to differentiated cells. This increase was even more profound on day 5 (61%±0.3%; $p<0.001$) and day 6 (157%±1.8%; $p<0.001$). Whereas there was no difference in formazan production between NGF treated PC12 cells at day 3 (0.48±0.01) and day 4 (0.55±0.02), 5 (0.52±0.02) or 6 (0.45±0.02). The data indicated NGF-treated cells ceased proliferation and terminally differentiated by day 3.

Immunohistochemical detection of tyrosine hydroxylase

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of catecholamines and is often used as a marker for differentiated PC12 cells. To confirm NGF-treated PC12 cells had undergone differentiation, immunohistochemical detection of TH was carried out and the amount of staining was compared between undifferentiated and differentiated cells. As shown in Fig 3-3A, there was little staining in undifferentiated PC12 cells. However, intense TH immunoreactivity was observed 3 days after PC12 cells were exposed to NGF in particular those had neurite extensions (Fig3-3B and Fig3-3C). This suggests PC12 cells had differentiated into neuronal-like cells expressing tyrosine hydroxylase.

Taken together, it demonstrates that undifferentiated PC12 cells accomplished differentiation by day 3 since addition of 100ng/ml NGF. Therefore, all the experiments using differentiated PC12 cells were carried out on day 3.

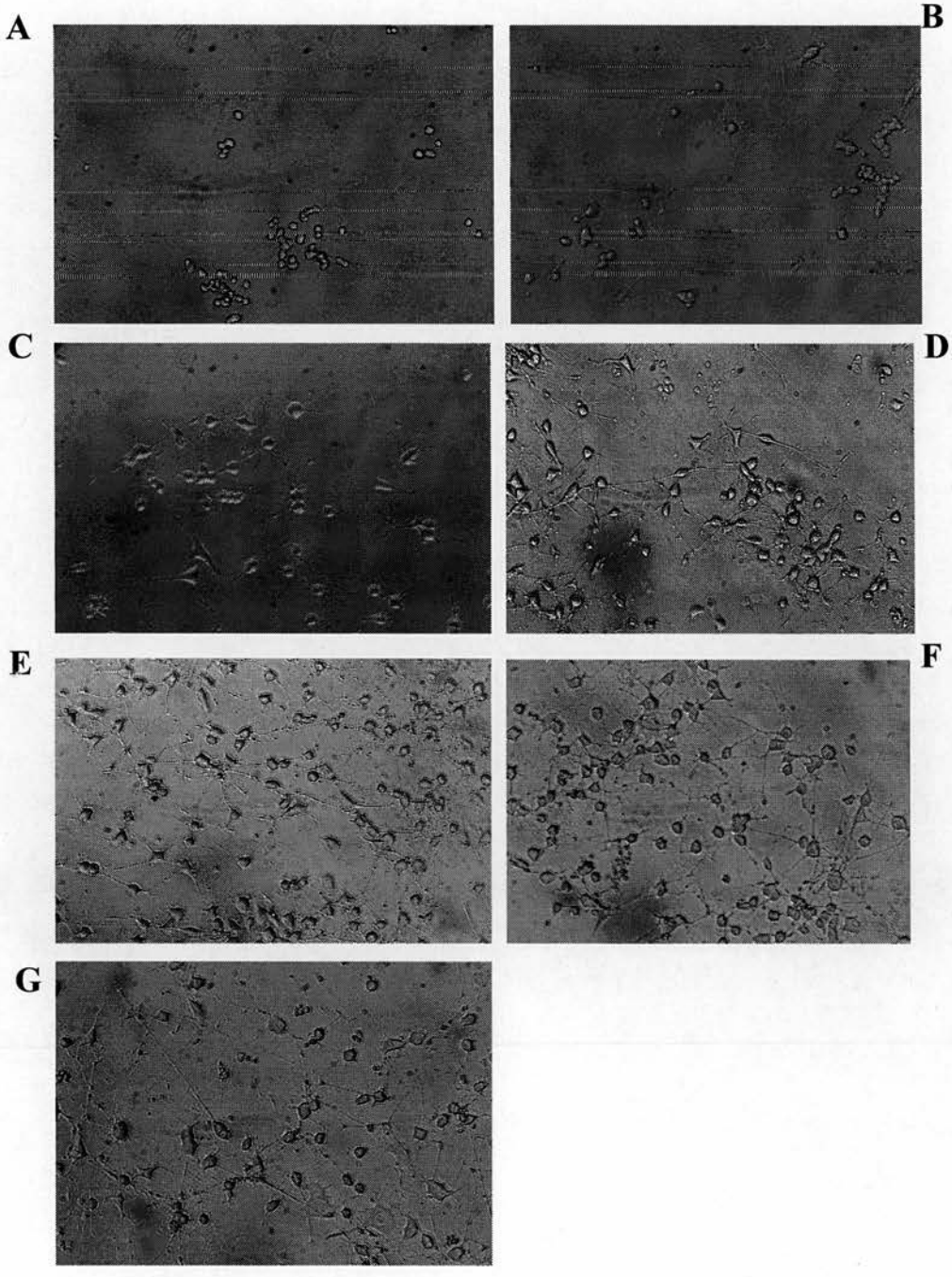


Fig 3-1. Progression of morphological changes of PC12 cells with exposure to NGF for 6 days. (A) Undifferentiated PC12 cells were seed on day 0. By day 1 cells had begun to sprout neurites and by day 3 were morphologically distinct showing cell-cell connections (B)-(D). This morphology was maintained until day 6 (E)-(G).

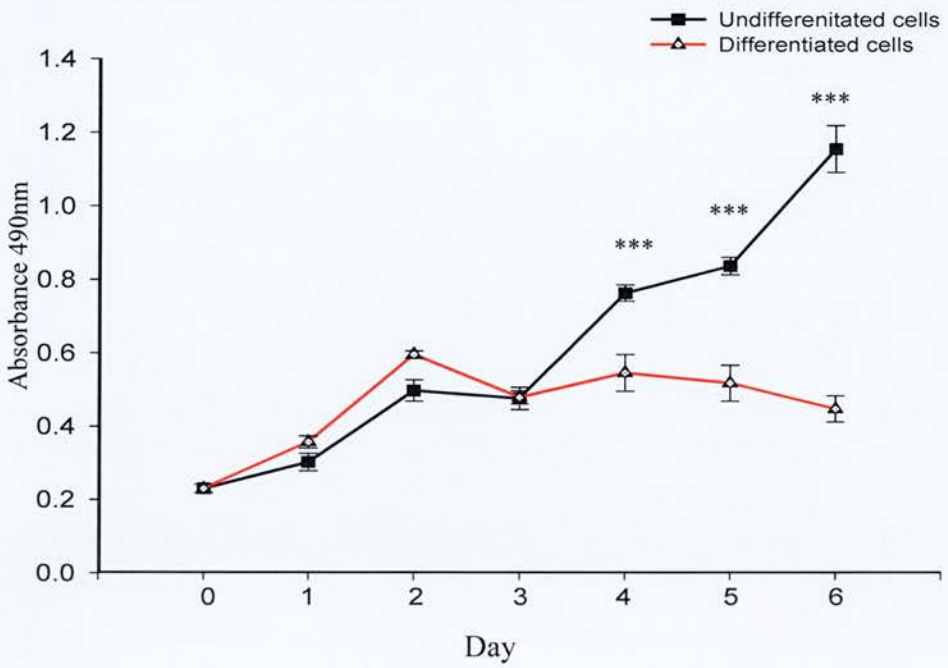


Fig 3-2. Rate of cell proliferation in PC12 cells incubated in the presence or absence of NGF, measured by MTS assay. Undifferentiated PC12 cells were exposed to 100ng/ml NGF for 6 days, with NGF replenished on 3rd day. Cells exposed to NGF showed an increase in absorbance between day0 and day3, but this was not further increased after day4. Undifferentiated cells showed a continuous increase in absorbance over the timescale measured. Values represent the mean ± S.E.M. ***$p<0.001$. n=4 independent experiments with each timepoint performed in triplicate.

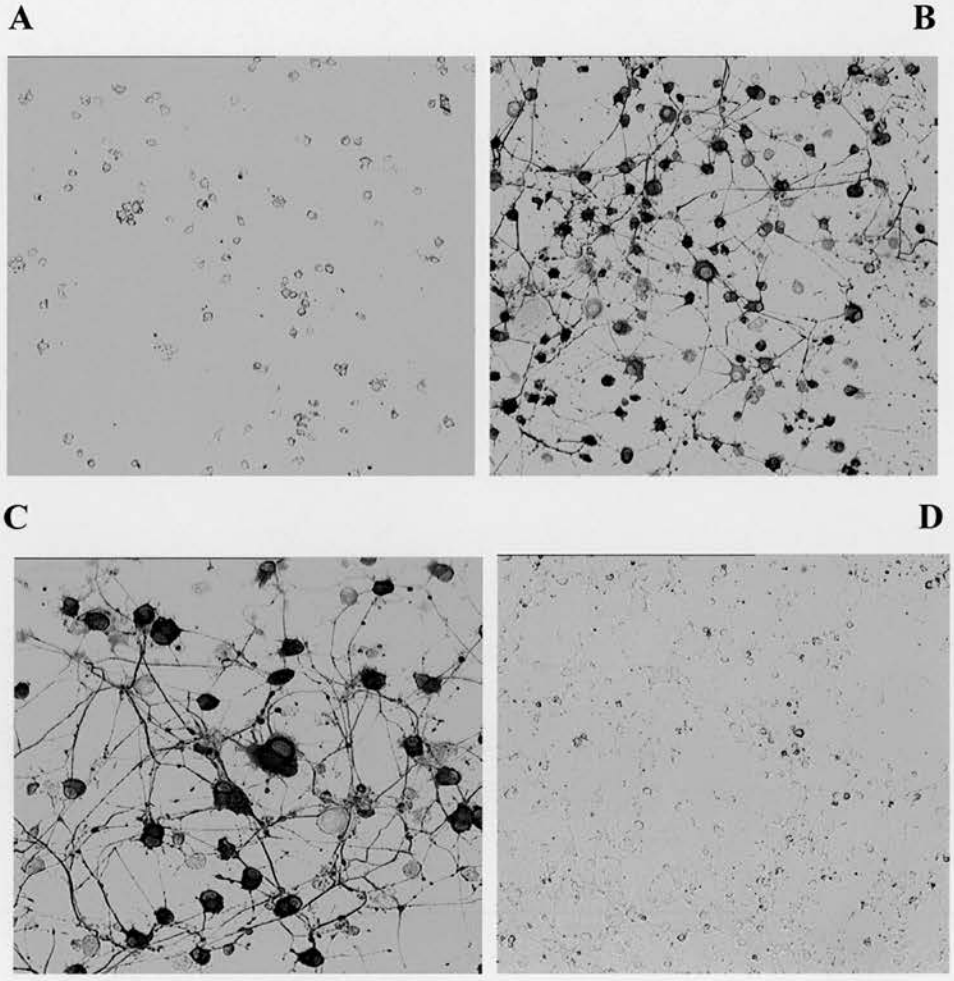


Fig 3-3. Immunohistochemical staining for tyrosine hydroxylase(TH) in undifferentiated and differentiated PC12 cells. (A) Undifferentiated PC12 cells with mouse anti-TH monoclonal antibody (1:500 dilution). (B) Differentiated PC12 cells with mouse anti-TH monoclonal antibody (1:500 dilution), ×20 times magnification (C) Differentiated PC12 cells with anti-TH monoclonal antibody (1:500 dilution), ×20 magnification. (D) Negative control: differentiated PC12 cells incubated without mouse anti-TH monoclonal antibody.

3.2.1.2 Selection of cell stressors

Previous work had demonstrated that MR induction occurred both *in vitro* when primary neuronal cultures were exposed to sub-lethal concentrations of staurosporine and *in vivo* in hippocampal neurons when animals had been subjected to hypothermic transient global cerebral ischaemia (Macleod *et al.* 2003). Therefore to determine how these insults might regulate MR, their effects on alternate MR promoter activity were investigated in both undifferentiated and differentiated PC12 cells. Ischaemia was mimicked *in vitro* by oxygen-glucose deprivation (OGD). The effects of OGD and hypothermia at 33°C alone and in combination (OGD&hypothermia) were studied. Both oxidative stress and hypersecretion of glutamate contribute to ischaemic damage therefore the effects of hydrogen peroxide (H_2O_2) and glutamate in the form of NMDA on MR regulation were also investigated.

3.2.1.3 Dose (time)-dependent effects of cellular stressors on cell viability

To determine the most suitable concentration or exposure time of each cell stressor for subsequent use in the promoter activity assays, dose- or time-response curves were performed. Release of lactate dehydrogenase (LDH) was used as the measure of cell damage. The concentration or time of exposure causing 20% of maximal cellular LDH release (representing sub-lethal challenge) was determined for each of the cell stress conditions.

Staurosporine

Undifferentiated and differentiated PC12 cells were exposed to staurosporine (10^{-9}M to 10^{-5}M) for 24 hours and the amount of LDH released into the culture medium was measured. Values were calculated as a percentage of total LDH,

expressed relative to non-stressed controls (defined as 0) and plotted using a Sigmoidal dose-response equation. As shown in Fig3-4 (A), in undifferentiated PC12 cells, 10uM staurosporine caused 65% LDH release in undifferentiated cells whereas this concentration caused 100% LDH release in differentiated cells. The EC_{20} was calculated to be 10nM in undifferentiated cells (correlation coefficient r^2=0.8185) and 3nM in differentiated cells (r^2=0.8484). As illustrated in Fig 3-4, the dose-response curve for differentiated cells was shifted to the left with respect to undifferentiated cells. However, there was no significant difference between the two dose-response curves (F=1.49, p=0.167, two way ANOVA), suggesting that in fact differentiated PC12 cells were no more susceptible to staurosporine than undifferentiated cells.

Hydrogen peroxide

Undifferentiated and differentiated PC12 cells were exposed to H_2O_2 (10uM-10mM) for 24 hours and data was expressed as described above. As shown in Fig 3-5, maximum cell damage (100% LDH release) was induced at 1mM (10^{-3}M) in undifferentiated cells and 3mM in differentiated cells. The EC_{20} calculated by curve fit was 100uM in undifferentiated cells (r^2=0.9710), and 300uM in differentiated cells (r^2=0.9574). In addition, the dose-response curve for undifferentiated cells was shifted significantly to the left with respect to differentiated cells (F=18.612, p<0.001, two-way ANOVA), indicating that undifferentiated cells were more susceptible to damage caused by hydrogen peroxide.

NMDA

Undifferentiated and differentiated PC12 cells were exposed to a range of concentrations [10^{-5}M to 10^{-2}M] of NMDA and the amount of LDH released was

measured 24 hours later. Results showed that there was no difference in the amount of LDH in the culture medium between undifferentiated, differentiated cells and vehicle treated cells at any concentration of NMDA. Therefore, the experiments determining the effects of NMDA on promoter activity were not carried out.

OGD

Undifferentiated PC12 cells were cultured in glucose-free DMEM medium in a hypoxic chamber (1% O_2, 5% CO_2 and 94% N_2) at 37°C over 6 different time points (1, 2, 4, 6, 12 and 24 hours). Cells were returned to normal culture conditions of glucose and oxygen in a standard cell culture incubator for the remaining 24 hours. As shown in Fig 3-7(A), for undifferentiated cells, compared to the control, there was a significant increase in LDH release at 4 (5.76%±0.66%, $p<0.01$) and 6 hours (5.09%±0.64%, $p<0.01$) but not at 12 or 24 hours.

The effects of OGD on the extent of LDH release in differentiated PC12 cells were assessed under two different conditions: either in the presence (+NGF) or absence of NGF (-NGF). As show in Fig 3-7(B), basal LDH release was 11.5% in +NGF group and 31.7% in –NGF group. There was no significant difference in LDH release either in the –NGF or +NGF group at the 1, 2 or 4-hour time point compared to the 0-hour control group. However, exposure to OGD for 6 hours caused significantly increased cell damage to 36.2% ($p<0.05$) in +NGF group and 58% ($p<0.05$) in –NGF group respectively in comparison to the non-OGD control. And exposure to OGD for 24 hours caused a greater LDH release (50%; $p<0.01$) in differentiated cells in the +NGF group, whereas in the –NGF group this was not significantly different from the 6-hour timepoint.

The overall magnitude of LDH release in both +NGF and –NGF groups was

calculated to be approximately 25%. However, the level of LDH release in 0-hour control group was significantly higher in the –NGF group compared to the +NGF group ($p<0.001$), suggesting that withdrawal of NGF itself appears to affect the viability of the cells. To this end, the -NGF group was excluded from subsequent promoter activity to avoid any confounding effects of NGF withdrawal. Therefore, cells cultured in the presence of NGF and exposed to 6-hour OGD were the chosen conditions for MR promoter studies in both undifferentiated and differentiated cells.

Hypothermia and OGD&hypothermia

For the purposes of continuity with the OGD experiments, the effects of 6-hour exposure to hypothermia and OGD&hypothermia in undifferentiated and differentiated cells (+NGF) on MR promoter activity were also used.

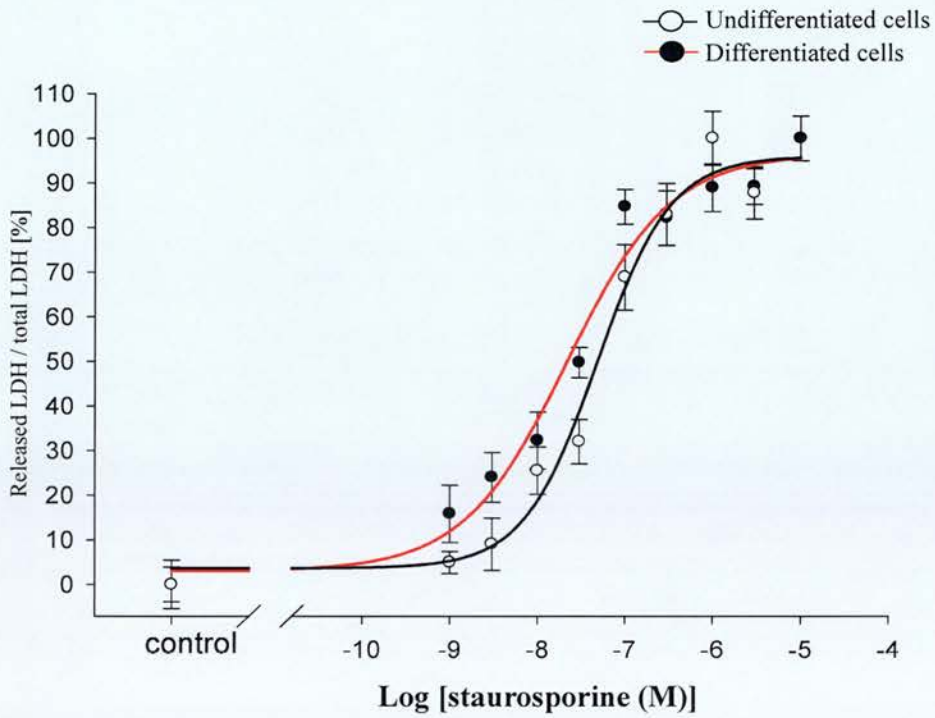


Fig 3-4. Dose-dependent effects of 24-hour exposure to staurosporine in undifferentiated and differentiated PC12 cells. Data was normalized with respect to the control group and plotted using the Sigmoidal dose-response equation. The EC_{20} was 10nM in undifferentiated cells and 3nM in undifferentiated cells. There was no significant difference between the two curves by two way ANOVA ($F=1.49$, $p=0.167$). Values represent the mean ± S.E.M. n=4 independent experiments, each point performed in triplicate.

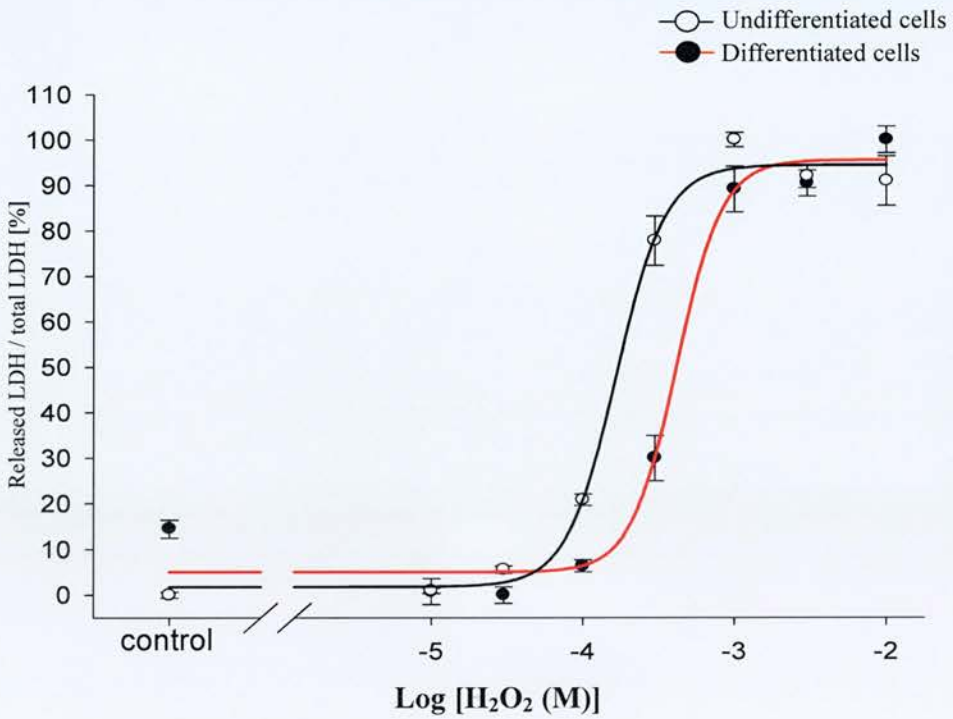


Fig 3-5. Dose-dependent effects of 24-hour exposure to hydrogen peroxide in undifferentiated and differentiated PC12 cells. Data was normalized with respect to the control group set and plotted using a Sigmoidal dose-response equation. The EC_{20} was 100uM in undifferentiated cells and 300uM in differentiated cells. There was a significant difference between the two curves by two way ANOVA ($F=18.612$, $p<0.001$). Values represent the mean ± S.E.M. n=4 independent experiments, each performed in triplicate.

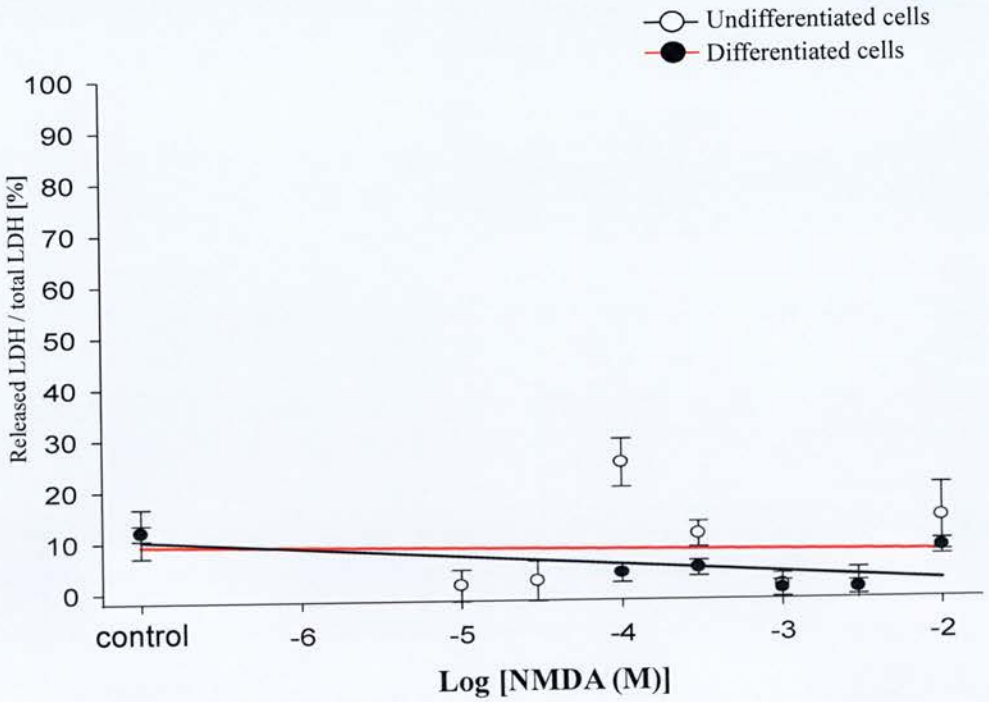


Fig 3-6. Dose-dependent effects of 24-hour exposure to NMDA in undifferentiated and differentiated PC12 cells. Data was normalized with respect to control group and plotted using the Sigmoidal dose-response equation. There was no effect of NMDA on LDH release. Values represent the mean ± S.E.M. n=4 independent experiment, each performed in triplicate.

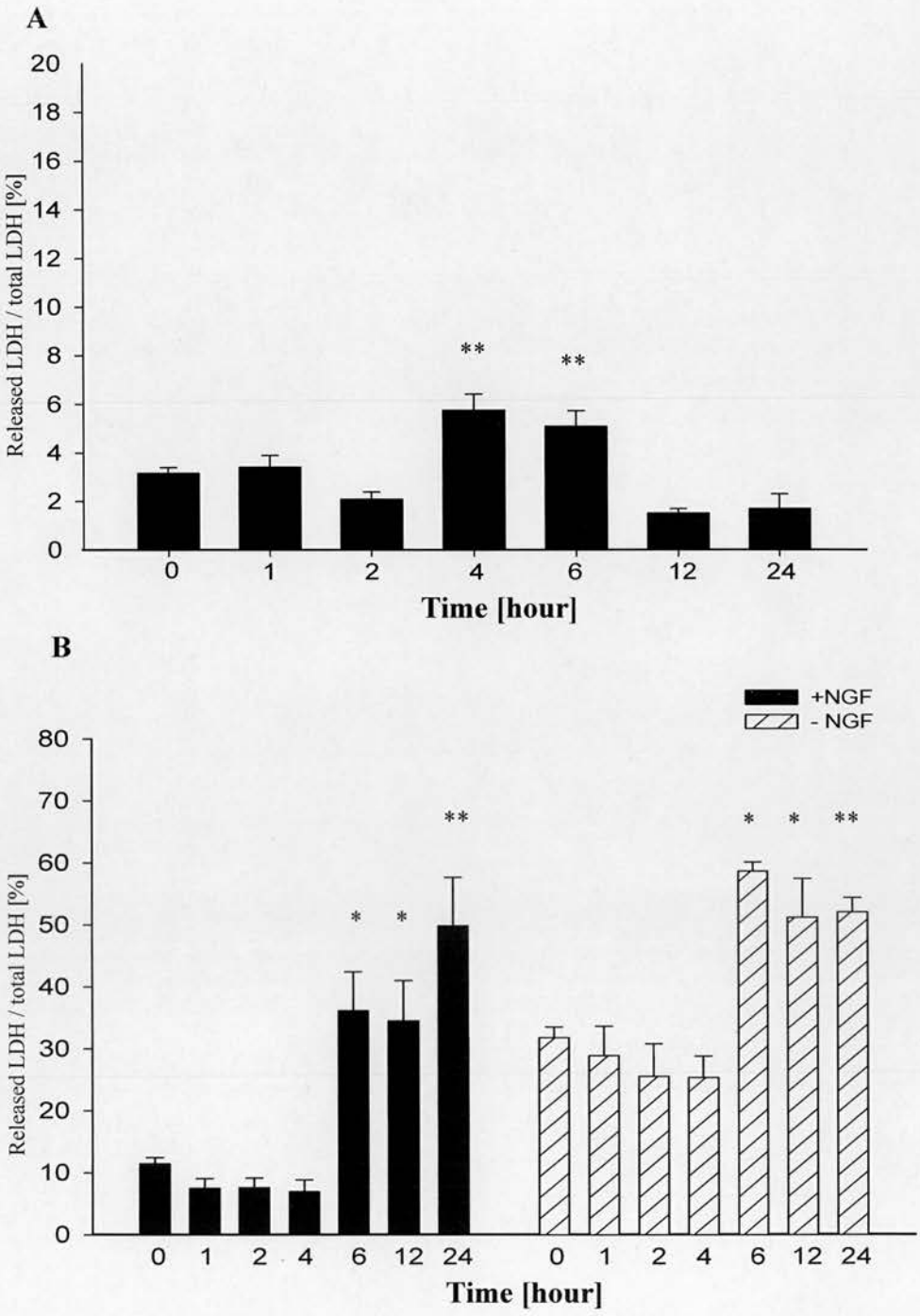


Fig 3-7. Time-course for OGD-induced LDH release in undifferentiated (A) and differentiated PC12 cells (B). LDH release was significantly higher at 4-hour and 6-hour exposure to OGD in undifferentiated cells ($p<0.01$) compared to 0 hr. At 6hr LDH release was significantly higher in differentiated PC12 cells –NGF (55%) than +NGF (35%) Values represent the mean ± S.E.M. *$P<0.05$; **$P<0.01$ vs 0 hour control. n=6 independent experiments, each performed in triplicate.

3.2.2 Generation of MRβ and MRγ promoter constructs

The recombinant plasmid pMRα containing a 1.5kb fragment of the MRα promoter was generated by Dr. Maija Castren, University of Kupio, Finland (Castren *et al.* 1993). To generate the plasmids pMRβ and pMRγ, the putative promoter fragments of MRβ and MRγ were amplified from rat genomic DNA respectively by PCR using high-fidelity DNA polymerase. PCR products were visualized by agarose gel electrophoresis and fragments generated were of the correct predicted sizes (MRβ = 300bp; γ = 1.7kb) as shown in Fig 3-8.

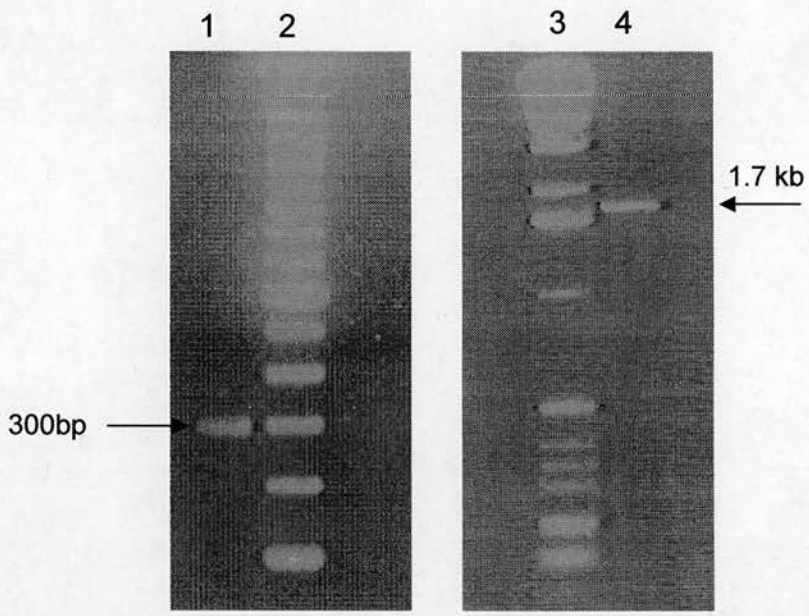


Fig 3-8. PCR amplification of the MRβ and MRγ promoter fragments. Lane 1 shows the MRβ PCR product at the predicted size of 300bp and lane 4 shows the 1.7kb MRγ PCR product. Lane 2 = 100bp ladder; lane 3 = 1Kb ladder.

3.2.3 Effects of cellular stressors on alternate MR promoter activity in undifferentiated PC12 cells

Each promoter plasmid was co-transfected into undifferentiated PC12 cells with a β-galactosidase-encoding plasmid to enable normalization of the transfection efficiency. Promoter activity was calculated as a mean ratio of luciferase to β-galactosidase activity and expressed relative to the promoterless pGL-3 vector. All stressors with the exception of NMDA were tested for their effects on promoter activity at the EC_{20} derived from section 3.2.1.2.

3.2.3.1 Basal activity

The mean basal activity (taken from the whole experimental set) of the MRα promoter was 3.42±0.37 fold higher than the promoterless vector pGL3 in undifferentiated PC12 cells, whereas MRβ promoter displayed the lowest basal activity at 0.19±0.01 fold of pGL3 while MRγ was 1.75±0.13 fold greater than pGL-3. It is noteworthy that although the actual values from each experiment were different, the pattern was similar throughout (MRα>MRγ>MRβ). (Tab 3-1)

3.2.3.2 Effect of cellular stressors

As shown in Fig 3-9, 10nM staurosporine significantly increased MRα promoter activity (300%±33%, $p<0.001$) compared to vehicle but had no effect on either MRβ or MRγ promoter activity. Exposure of undifferentiated cells to hydrogen peroxide(Fig3-10), OGD(Fig3-11), hypothermia(Fig3-12) or OGD&hypothermia (Fig3-10) had no effect on MRα, MRβ or MRγ promoter activity.

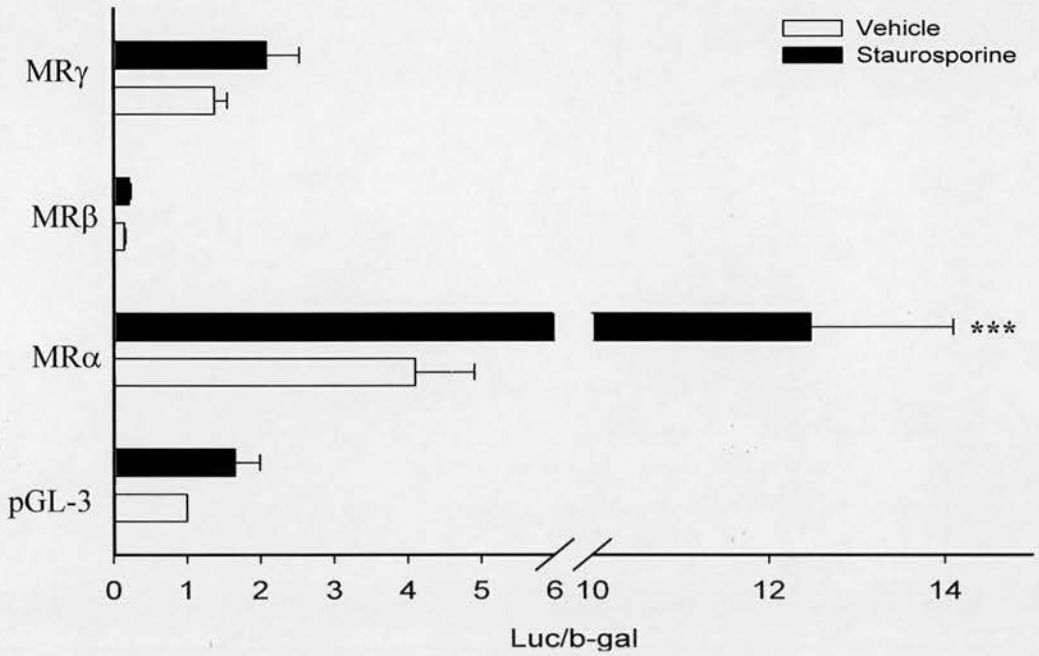


Fig 3-9. Effect of 10nM staurosporine on MR promoter activity in undifferentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs and promoter activity of each construct was determined and expressed as relative luciferase activity. MRα promoter activity was significantly increased in the presence of staurosporine (300% ±33%, $p<0.001$) compared to vehicle, while there were no significant changes in MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. ***$p<0.001$. n=6 independent experiments performed in triplicate

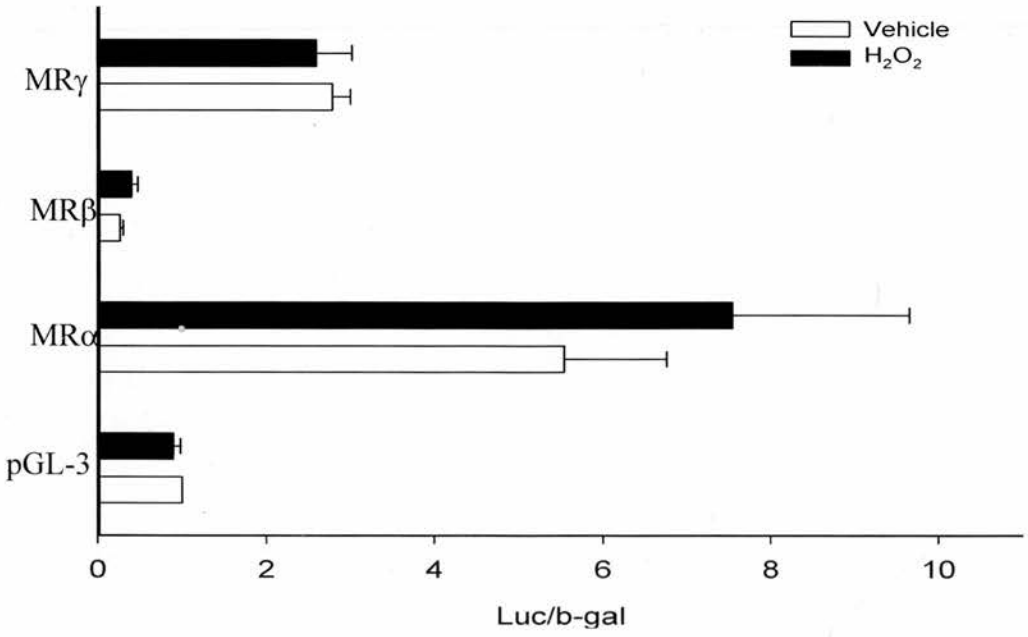


Fig 3-10. Effect of 100uM H_2O_2 on MR promoter activity in undifferemtiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs and promoter activity of each construct was determined and expressed as relative luciferase activity. No significant changes were observed for MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=5 independent experiments each performed in triplicate

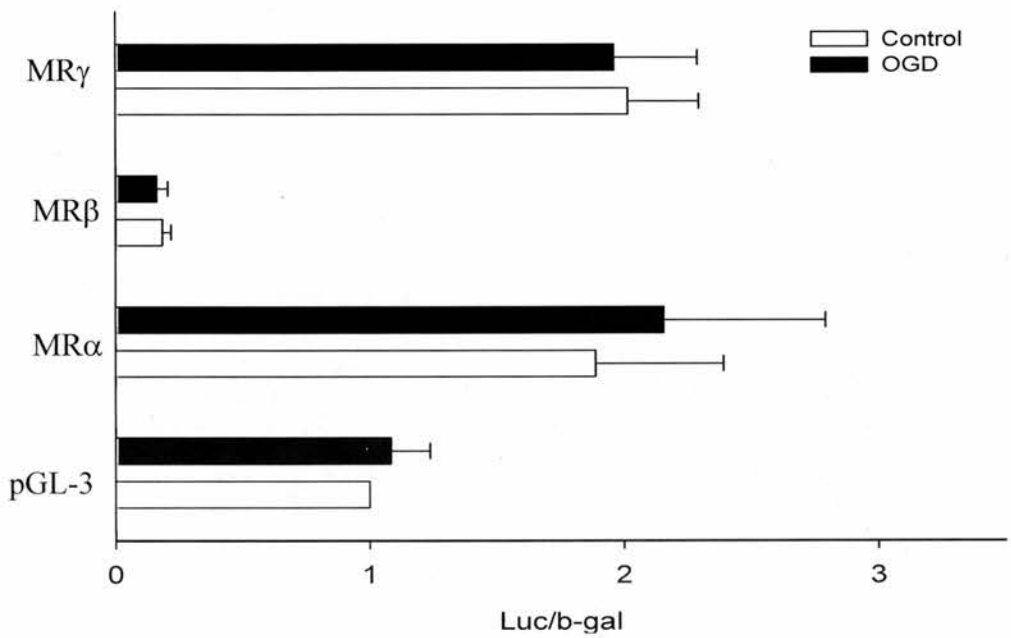


Fig 3-11. Effect of OGD (6-hour) on MR promoter activity in undifferentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs and promoter activity of each construct was determined and expressed as relative luciferase activity. No significant changes were observed for MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=6 independent experiments, each performed in triplicate.

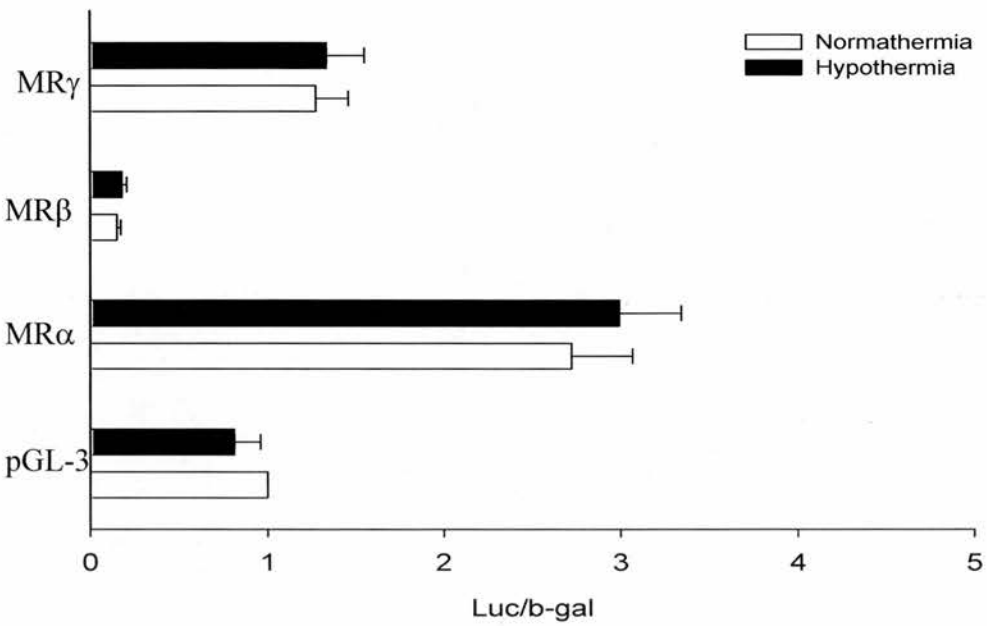


Fig 3-12. Effect of hypothermia (6-hour) on MR promoter activity in undifferentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs and promoter activity of each construct was determined and expressed as relative luciferase activity. No significant changes were observed for MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=6 independent experiments each performed in triplicate.

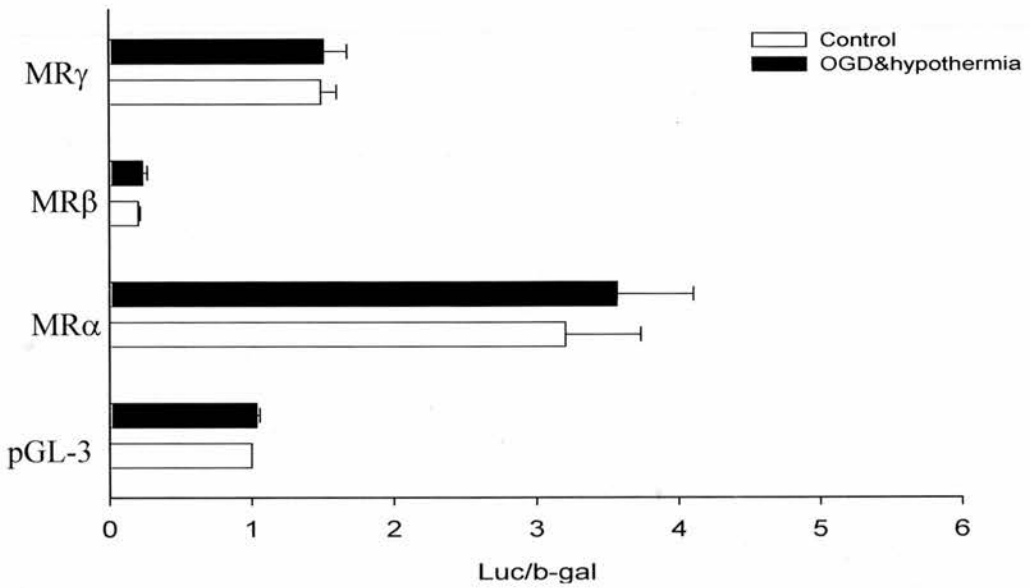


Fig 3-13. Effect of OGD&hypothermia (6-hour) on MR promoter activity in undifferentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs and promoter activity of each construct was determined and expressed as relative luciferase activity. No significant changes were observed for MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=6 independent experiments, each performed in triplicate.

3.2.4 Effects of cellular stressors on alternate MR promoter activity in differentiated PC12 cells

The efficiency of transfection in differentiated cells was found to be extremely low in pilot studies, therefore to circumvent this problem, undifferentiated cells were transiently transfected with each promoter construct and then exposed to NGF for differentiation. Expression of constructs was stable for at least 7 days following introduction of NGF (data not shown). Cells were subjected to the different cell stress conditions on day 4 of differentiation and promoter activity was measured 24 hours later as described for undifferentiated cells.

3.2.4.1 Basal activity

The mean basal promoter activity was calculated from all of the experiments performed. Compared to the activity of the pGL-3 promoterless vector, MRα promoter displayed the highest basal activity (17.68±1.5 fold), followed by MRγ (8.49±0.96 fold) and MRβ (1.27±0.17 fold) in differentiated cells. This pattern of basal activity was in accordance with undifferentiated cells. Furthermore, the basal activity of each promoter in differentiated PC12 cells was also significantly increased compared to undifferentiated cells ($p<0.001$). (Table 3-1).

Table 3-1 Basal promoter activity of MRα, MRβ and MRγ in undifferentiated and differentiated PC12 cells compared to pGL-3

	Undifferentiated cells (Fold)	Differentiated cells (Fold)	P value
MRα	3.42 ± 0.37	17.68 ± 1.5	<0.001
MRβ	0.19 ± 0.01	1.27 ± 0.17	<0.001
MRγ	1.75 ± 0.13	8.49 ± 0.96	<0.001

3.2.4.2 Effect of cellular stressors on MR promoter activity in differentiated PC12 cells

Exposure to staurosporine (3nM) for 24 hours caused a significant increase in MRα promoter activity compared to vehicle treated group (215.4%±22%, $p<0.05$) (Fig 3-14). However, staurosporine had no effect on either MRβ or MRγ promoter activity. As with undifferentiated PC12 cells, hydrogen peroxide (300uM) (Fig 3-15), OGD (6-hour) (Fig 3-16) or hypothermia (6-hour) (Fig 3-17) did not induce significant changes in MRα, MRβ or MRγ promoter activity compared to vehicle group.

However, exposure to OGD&hypothermia significantly increased MRα promoter by 51% ($p<0.001$) compared to the control group (Fig 3-18). The promoter activity of MRβ was also increased significantly by 85% ($p<0.05$) while MRγ promoter activity was not affected by OGD&hypothermia at any level.

Overall, these results show that there is differential regulation of MR promoters in response to different types of cell stress in differentiated PC12 cells.

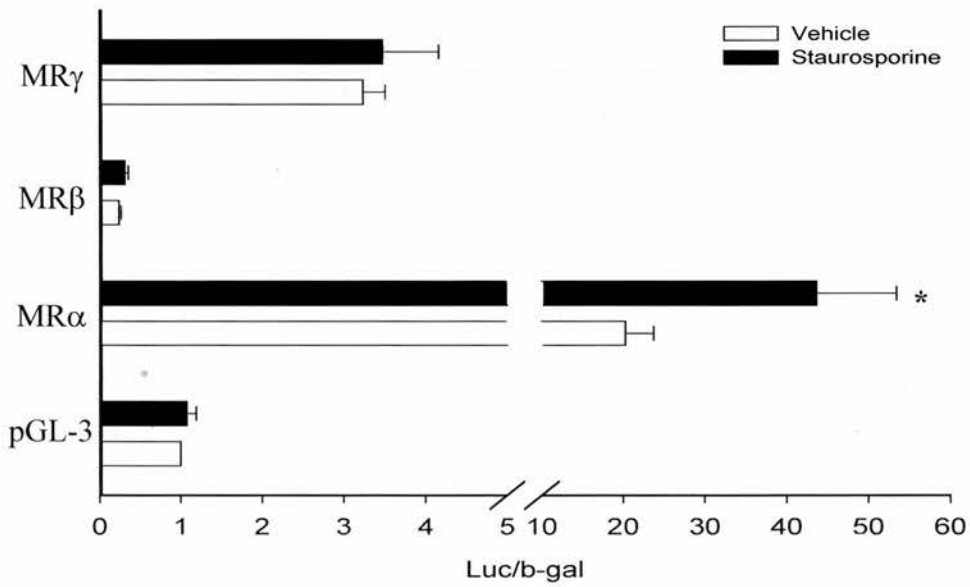


Fig 3-14. Effect of 3nM staurosporine on MR promoter activity in differentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs, differentiated for 3 days and promoter activity of each construct in the presence of staurosporine was determined and expressed as relative luciferase activity. MRα promoter activity was increased by 212.4%±31.4% ($p<0.05$) compared to vehicle but neither MRβ nor γ promoter activity was affected. Values represent the mean ± S.E.M. *$p<0.05$. n=6 independent experiments each performed in triplicate.

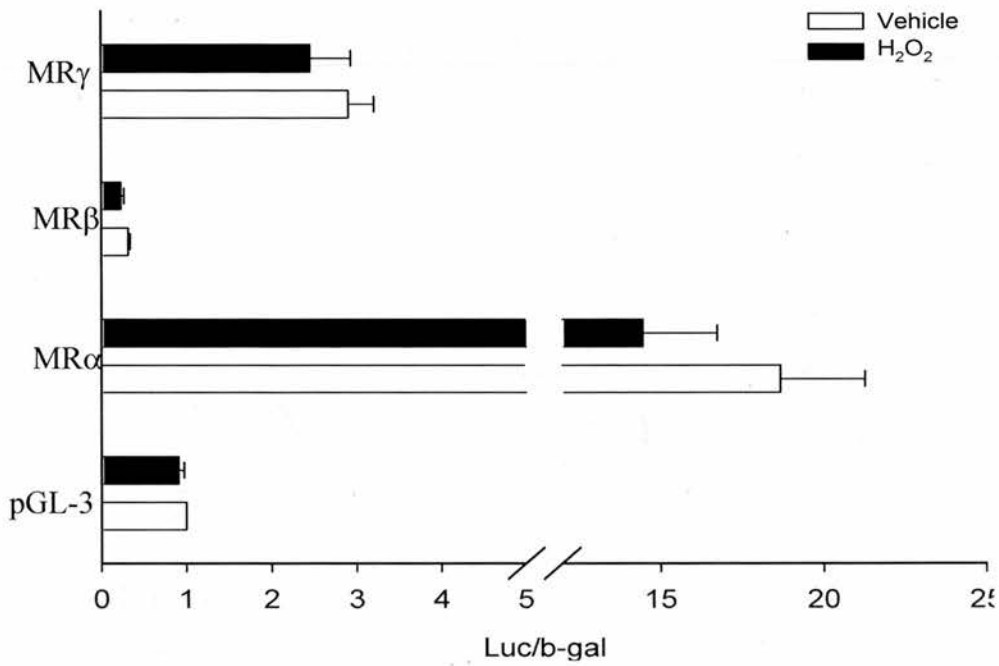


Fig 3-15. Effect of H_2O_2 (300uM) on MR promoter activity on differentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs, differentiated for 3 days and promoter activity of each construct in the presence of H_2O_2 was determined and expressed as relative luciferase activity in differentiated PC12 cells. No significant changes were observed for MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=5 independent experiments, each performed in triplicate.

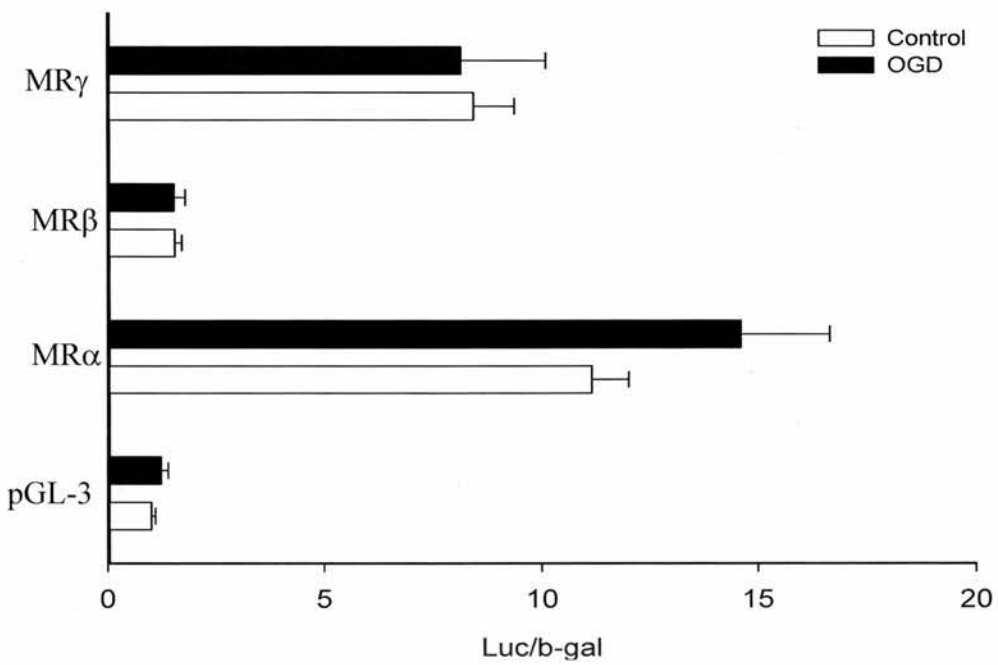


Fig 3-16. Effect of OGD (6-hour) on MR promoter activity in differentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs, differentiated for 3 days and promoter activity of each construct exposed to OGD was determined and expressed as relative luciferase activity. There were no significant changes in MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=4-10 independent experiments, each performed in triplicate.

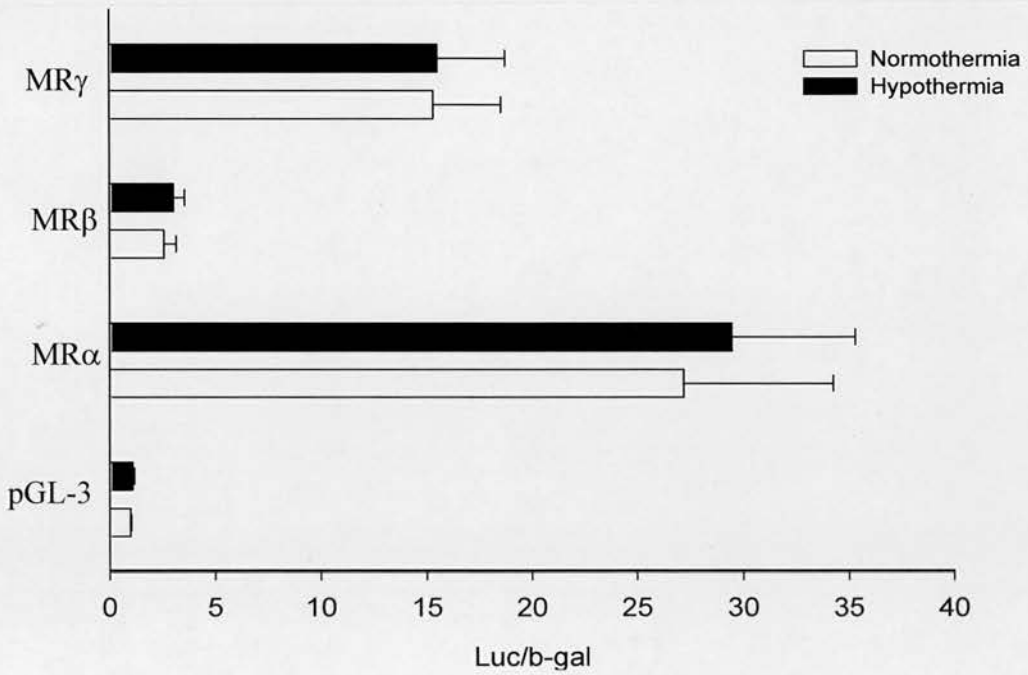


Fig 3-17. Effect of hypothermia (6-hour) on MR promoter activity in differentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs, differentiated for 3 days and promoter activity of each construct exposed to hypothermia was determined and expressed as relative luciferase activity. There were no significant changes in MRα, MRβ or MRγ promoter activity. Values represent the mean ± S.E.M. n=5 independent experiments, each performed in triplicate.

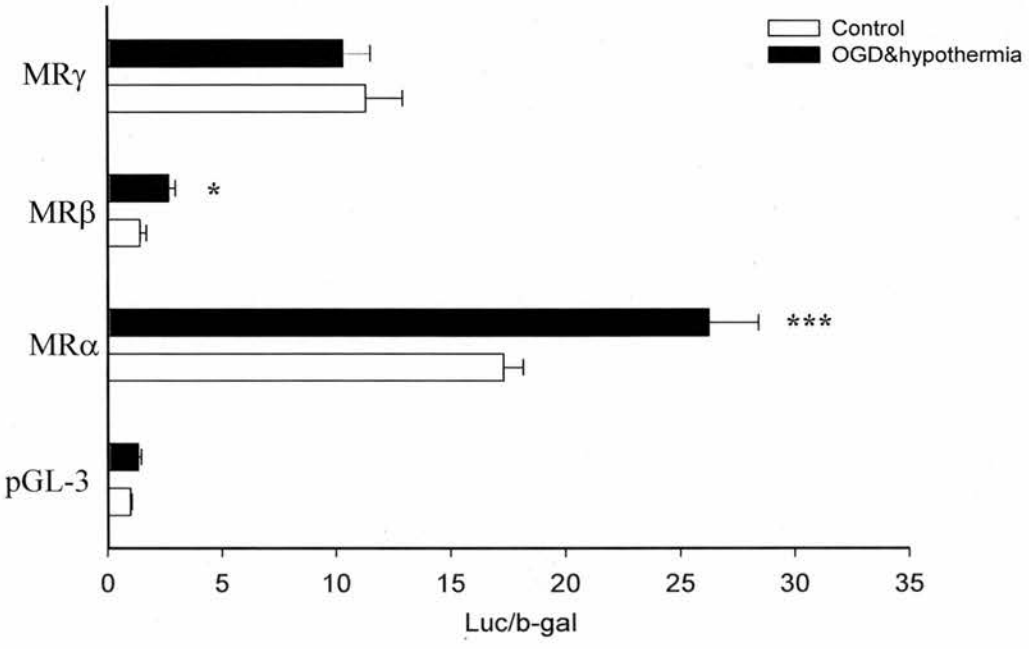


Fig 3-18. Effect of OGD&hypothermia (6-hour) on MR promoter activity in differentiated PC12 cells. PC12 cells were transiently transfected with MRα, MRβ or MRγ promoter constructs, differentiated for 3 days and promoter activity of each construct exposed to OGD&hypothermia was determined and expressed as relative luciferase activity. MRα promoter activity was significantly increased by 51% ($p<0.001$) and MRβ promoter activity was also significantly increased by 85% ($p<0.05$) compared to control. No changes were seen in MRγ promoter activity. Values represent the mean ± S.E.M. *$p<0.05$; ***$p<0.001$. n=5-9 independent experiments, each performed in triplicate.

3.3 Discussion

The aim of this chapter was to determine if MR might be differentially regulated at the promoter level in response to different types of cell stress and if this regulation was specific to cell-type. The results are summarized in Table 3-2.

Table 3-2. Effect of different types of cell stress on MRα, MRβ and MRγ promoter activity: comparison between undifferentiated and differentiated PC12 cells

	Undifferentiated PC12			Differentiated PC12		
	MRα	MRβ	MRγ	MRα	MRβ	MRγ
Staurosporine	↑	↔	↔	↑	↔	↔
H_2O_2	↔	↔	↔	↔	↔	↔
Hypothermia	↔	↔	↔	↔	↔	↔
OGD	↔	↔	↔	↔	↔	↔
OGD&hypothermia	↔	↔	↔	↑	↑	↔
↔ no change; ↑ significant increase						

Data in this chapter demonstrated that there is indeed differential regulation of MR variants in response to cell stress. This up-regulation was not a generic response since only some but not all types of cellular stressors investigated caused induction of MR variant promoter activity.

Staurosporine is commonly used as a general apoptosis-inducing agent (Jacobsen *et al.* 1996; Wiesner *et al.* 1996; Fitzgerald *et al.* 2007). 10nM staurosporine caused a significant increase in MR expression in primary cortical culture (Macleod *et al.* 2003). In this experiment, MRα promoter activity was significantly increased in response to staurosporine, suggesting the total MR induction in primary cortical culture was primarily mediated by MRα. In addition,

the effect of staurosporine on MR expression does not depend on a specific cell-type since this was observed in both undifferentiated and differentiated cells.

OGD&hypothermia significantly increased both MRα and MRβ promoter activity in differentiated PC12 cells, whereas neither OGD nor hypothermia alone had effects on any of the MR promoters. These observations corroborate previous findings that MR expression can be induced by hypothermic but not normothermic ischaemia *in vivo* (Macleod *et al.* 2003). It is known that mild to moderate hypothermia generally reduces ischaemic damage in the brain (Busto *et al.* 1989; Buchan *et al.* 1990; Baker *et al.* 1995). The exact mechanisms of hypothermic neuroprotection is still not clear, but the expression of some genes are altered by hypothermia (Ohta *et al.* 2007), some of which are known to be neuroprotective, indicating that hypothermia itself triggers an endogenous survival response. This work suggests that MR induction may form part of the neuroprotective effects of hypothermia. The differential regulation of transcription through alternate promoter usage in the context of brain injury is not unique to MR, since it is known that other genes encoding protective proteins such as BDNF also undergo differential regulation in response to cerebral ischaemia in hippocampal and cortical neurons through stimulation of at least two different promoters (Tsukahara *et al.* 1998).

More importantly, the induction of MRα and MRβ promoter activity by OGD&hypothermia was largely confined to neuronal-like cells since this was only observed in differentiated PC12 cells. This demonstrates that such induction of MR is subjected not only to the type of cell stress but also to the type of cell. While the MRα promoter responds to more varied stimuli in different cell types, the MRβ promoter appears to be specifically activated in neuronal-like cells to neuronally relevant stimuli OGD&hypothermia. This has important implications

and identifies the MRβ promoter as a potential target for clinical interventions for conditions of cerebral ischaemia. The goal would be to enhance MR expression specifically in neurons, because the activation of MR in other cell type or tissue may be detrimental. For example activation of MR in the vasculature vessels leads to vascular remodelling (1.4.5.3) involving reduced lumen and outer diameters but normal vascular wall thickness (Dorrance *et al.* 2006) and causing hypertension which itself is a risk factor for ischaemia (Hilleman *et al.* 2004; Sokol *et al.* 2004). Moreover, increased MR signalling in the cardiac myocytes is associated with cardiac hypertrophy and fibrosis (Takeda *et al.* 2002). Although the MRβ promoter was not altered by OGD&hypothermia in undifferentiated cells, it will be necessary to carry out further studies to exclude the possibility that the MRβ is stimulated in other cell types by clinical relevant conditions of neuronal stress.

Interestingly, the MR promoters showed different basal activities both in undifferentiated and differentiated PC12 cells, displaying an order from the highest to lowest MRα>MRγ>MRβ. Only regulation of the MRα promoter has been reported in the literature, which showed an increased activity in response to corticosterone and progesterone in a rat neuroblastoma cell line (Castren et al. 1993; Castren et al. 1995; Castren et al. 1995), however there are no studies reported of the regulation of either the MRβ or MRγ promoter.

Since the distance between MR exon1β and exon1γ is only 300bp, the promoter fragment of MRβ was initially generated to span this short region. The basal promoter activity of MRβ was lowest of the three promoters in both undifferentiated and differentiated PC12 cells. This was probably attributable to lack of the necessary enhancers within this 300bp DNA fragment. A quick analysis of putative binding sites suggested that there was no TATA box or other classical transcriptional initiation sites located within this region and a transcriptional

Fig 3-19. Illustration of MRβ promoter region. Putative transcriptional factor binding sites are predicted using online software Genomatix (http://www.genomatix.de/). A transcriptional repressor is located at -185bp, as shown in square; +1: transcription initiation site. Underline: Exon.

repressor was found upstream at -185bp from the transcription initiation site (Fig3-19), which may also account for the low activity of MRβ promoter. 1.7kb rat MRγ promoter fragment showed a substantial basal activity, suggesting it was fair active in a biological context.

The basal promoter activity of each of MR variants in differentiated cells was significantly higher than undifferentiated cells (Tab 3-2). This implied all of MR variant promoters were switched on in neuronal-like cells but repressed to some extent in non-neuronal cells. This is not novel since some other genes have been reported to be differentially expressed in a cell type-specific manner, e.g. BDNF (Hohl *et al.* 2005). MRβ and MRγ mRNA levels were highly expressed in developing hippocampus but decline postnatally (Vazquez *et al.* 1998), suggesting an induction of its own promoter may be utilized. During the transition of phenotype of PC12 cells from undifferentiated to neuronal-like differentiated state, MR variant promoters may also be induced.

The PC12 cell line itself is a useful tool to compare the activity of the same promoter in two different cell types because of its ability to differentiate into neurite-bearing cells resembling mature sympathetic neurons (Greene *et al.* 1976; Greene 1978). These cells have been used extensively as a neuronal model for studying neuropathophysiological mechanisms (Rukenstein *et al.* 1991; Ferrari *et al.* 1993; Maroto *et al.* 1997; Levites *et al.* 2003; Qing *et al.* 2003), including those associated with ischemia (Tabakman *et al.* 2005; Hillion *et al.* 2006) and were used here to specifically test MR promoter activity due to the ease of transfection of promoter constructs. Primary neuronal cultures would be the ideal model but are notoriously difficult to transfect because they are terminally differentiated and indeed, initial pilot studies revealed that differentiated PC12 cells themselves were difficult to transfect. One way around this was to transfect the PC12 cells and then to differentiate them.

LDH is a stable cytosolic enzyme and released into culture medium when the integrity of cell membrane is damaged (Korzeniewski *et al.* 1983). Measurement of released LDH can directly reflect the severity of cellular damage quantitatively. However, it is not an ideal assay for measurement of apoptosis, since LDH is not an apoptotic marker protein. In this study, the optimal dose or time point for "stressing" cells was determined by measurement of cell damage (membrane integrity) instead of apoptosis. This is because cell damage is a better index reflecting the concept of "cellular stress", which ranges from the initial damage to membrane integrity to the final stage cellular death, whereas apoptosis is only one of those events.

6-hour OGD caused a significant increase in cell damage from basal level 11.49% to 35% in differentiated PC12 cells in the presence of NGF, suggesting differentiated cells were sensitive to this neuronal-specific insult. Although there

was a significant increase in undifferentiated cells at 4- and 6-hour exposure, the absolute percentage of cell damage was too small, at approximately 5-6%. It is assumed that this did not mount a profound stressful signalling, which might explain why OGD or OGD&hypothermia failed to induce any effect on MR variant promoters in undifferentiated cells. In addition, it is noteworthy that the PC12 cells do not model neurons entirely but lack certain intrinsic properties of neurons. For example, the overactivation of NMDA receptor has been stated as one of the major processes in ischaemic neuronal death *in vivo* and *in vitro* (Dingledine *et al.* 1999; Goebel *et al.* 1999; Lipton 1999). In this study, NMDA failed to achieve any level of cell damage in either undifferentiated or differentiated PC12 cells, which suggested this type of cell system still lacks some neuronal properties. The failure of NMDA may be attributed to the absence of functional NMDA receptor in PC12 cells (Gerlach *et al.* 2002; Edwards *et al.* 2007), despite the presence of subunits in PC12 cells (Sucher *et al.* 1993; Casado *et al.* 1996).

3.4 Concluding remarks

In conclusion, MR is differentially regulated at the promoter level by different conditions of neuronal stress which appears to depend on the cell type. The MRα promoter responds to both non-neuronal and neuronal-like stimuli whereas the MRβ promoter is specifically activated by neuronally relevant stimuli in neuronal-like cells. The MRγ promoter however does not respond to any of the cell stressors. As far as is known this is the first evidence for a mechanism regulating the MRβ promoter and suggests that regulation of MR via the MRβ promoter activity specifically serves to enhance neuronal survival. Thus targeting the MRβ promoter may form a useful strategy for the development of treatments

for cerebral ischaemia.

Chapter 4

MESSENGER RNA LEVELS OF THE MINERALOCORTICOID RECEPTOR VARIANTS IN PRIMARY CORTICAL CULTURE IN RESPONSE TO CELLULAR STRESSORS

4.1 Introduction

Results obtained from Chapter 3 show that different types of cell stress differentially affect the activity of the MR variant promoters and this is dependent on the differentiation status of the PC12 cells. While the promoter activity provides an indication of the changes in gene expression, the assay itself is artificial using a small portion of the actual promoter and enhancer region of a given gene which does not truly reflect changes in the abundance of the gene. Furthermore, although differentiated PC12 cells are considered a model of neuronal cells there are several limitations particularly as they are not derived from the central nervous system and do not possess all the characteristics of neuronal cells. Induction of total MR mRNA in response to cell stress (exposure to staurosporine) has been described previously (Macleod *et al.* 2003), however neither the effects of OGD, hypothermia or OGD&hypothermia on total MR mRNA nor their effects on MR variant mRNA expression have been explored.

Therefore this chapter considers the effect of different types of cell stress on MR variant mRNA expression in primary cortical neuronal culture. These cultures provide a more suitable model than differentiated PC12 cells because they are derived from the embryonic brain tissue and express endogenous MR. Therefore

the aims of this chapter were to investigate:

(i) Which MR variant mRNA level(s) is/are altered by cellular stressors in rat primary cortical culture.

(ii) If total MR expression is changed due to such altered MR variant(s) in response to the cellular stressors.

4.2 Results

4.2.1 Optimisation

Primary cortical cultures were generated as described in Materials & Methods and as with PC12 cells, experimental cell culture conditions were optimised by performing time-response curves. Given that changes in promoter activity were observed primarily in response to OGD&hypothermia and not to chemically-induced cell stress other than staurosporine, only the effects of OGD, hypothermia, OGD&hypothermia and staurosporine on MR variant mRNA expression were investigated by Ribonuclease Protection Assay (RPA).

4.2.1.1 Characterization of primary cortical culture

To determine the proportion of neurons in a typical primary cortical culture preparation, immunohistochemistry was performed using an antibody against the neuronal marker microtubule-associated protein 2 (MAP-2) and an antibody against the glial marker glial-fibrillary acidic protein (GFAP). As shown in Fig 4-1, a greater proportion of cells were stained with MAP-2 (Fig 4.1A) compared to GFAP (Fig. 4.1B). To determine the proportion of neurons to glia, the number of stained neurons or glia was divided by the total number of the cells in any given culture well. Unstained cells were clearly visible under phase-contrast

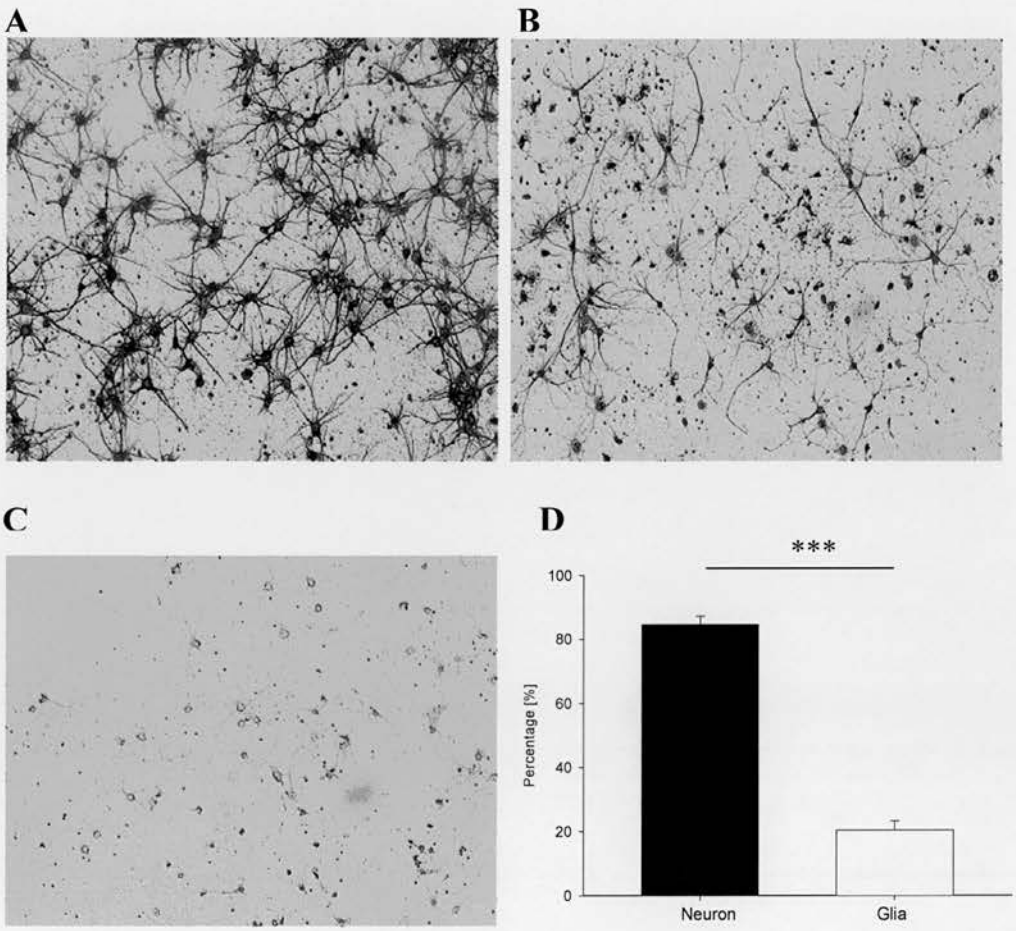


Fig 4-1. Immunohistochemical staining for MAP-2 (A) and GFAP (B) in primary cortical culture at day14. Incubation in the absence of primary antibody (MAP2 or GFAP) served as negative control (C). Stained cells were counted and expressed as a percentage of the total cells for each given field. Values represent the mean ± S.E.M , ***$p<0.001$, n = 4 independent experiments with each performed in duplicate. Magnification X 20.

microscopy. As shown in Fig 4-1(D) a typical preparation of primary cortical culture is predominantly composed of approximately 80% neurons and 20% glia.

4.2.1.2 Time-dependent effects of cellular stressors on cell viability

The effects of OGD and OGD&hypothermia over 24 hours on cell viability in primary cortical culture were assessed by measurement of the amount of LDH release as described in Chapter 3 to identify a suitable exposure time which caused sub-lethal damage (~20% LDH release). Both the effects of OGD with and without a period of recovery to a total of 24 hours which mirrors so-called reperfusion *in vivo*, on LDH release were evaluated. The concentration of staurosporine used was 10nM since this concentration has previously been shown to induce total MR mRNA and protein (Macleod *et al.* 2003).

OGD without recovery

Primary cortical neurons were cultured in glucose-free DMEM medium in a hypoxic chamber (1% O_2, 5% CO_2 and 94% N_2) at 37°C over 6 different time points (1, 2, 4, 6, 12 and 24 hours). Cell damage was measured immediately after the end of each time point. As shown in Fig 4-2, the basal level of LDH release in primary cortical culture was 12.37%±1.85%. Both 4-hour and 6-hour exposure to OGD caused a significant increase in LDH release (23.28%±3.45%, $p<0.05$; 27.63%±2.35%, $p<0.01$) compared to the controls. The greatest amount of LDH released was obtained after 24-hour exposure to OGD (54.49%±4.85%, $p<0.001$). Since there was no significant difference between 4-hour and 6-hour time points ($p=0.317$), the latter was chosen as the exposure time for OGD insult which allowed a longer period for the potential changes of the MR variant transcripts.

6-hour OGD with recovery

In Chapter 3, PC12 cells were returned to normal culture conditions following exposure to OGD. Accordingly, the effects of OGD followed by recovery on LDH release were also assessed in primary cortical culture. Based on the optimal exposure time for OGD, 5 time points (0.5, 1, 2, 4 and 18 hours) for recovery were chosen following 6-hour OGD treatment. In Fig 4-3, a 30-minute recovery period caused 41%±3.33% LDH release, significantly higher than 6-hour OGD without recovery (27.63%±2.35%, $p<0.05$). This became more profound with the longer recovery time such that an 18-hour recovery period resulted in 83.51%±1.5% LDH release. This suggests that any amount of recovery causes additional damage and therefore might confound any changes seen with MR expression and in line with previous experiments (Macleod *et al.* 2003), therefore RNA was extracted immediately from primary cortical culture for analysis following a 6-hour exposure to OGD.

OGD&hypothermia

The effects of different periods of exposure to OGD&hypothermia on LDH release was also evaluated in primary cortical culture. Basal LDH release in controls was 13.68%±1.59%. Exposure to OGD&hypothermia over 4 time points (1-6 hours) did not result in any significant changes compared to controls. However, 16-hour (22.35%±0.38%, $p<0.01$) and 24-hour (23.22%±0.31%, $p<0.001$) exposure to OGD&hypothermia caused a significant increase in LDH release in comparison to controls. These results suggest that hypothermia given at the same time as OGD as expected exerts a protective effect. In line with the OGD experiments, for subsequent experiments primary cortical cultures were exposed to a 6-hour period of OGD&hypothermia and then harvested immediately for mRNA analysis.

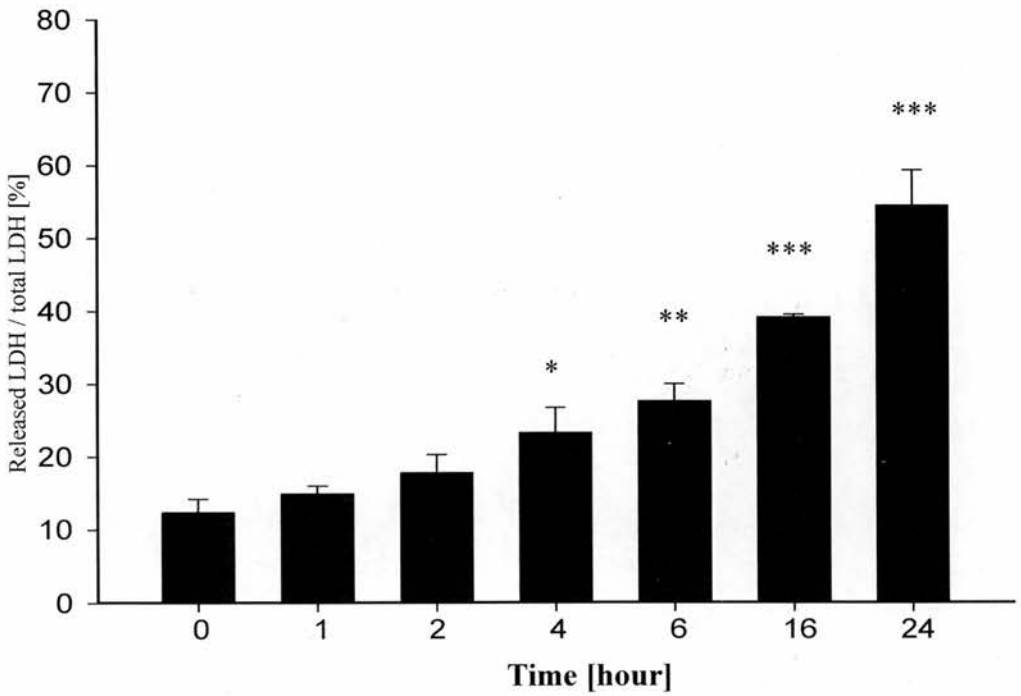


Fig 4-2. Time-course of OGD-induced LDH release in primary cortical culture. 6 time points were selected and LDH was immediately measured after OGD exposure. LDH release was significantly increased from 4-hour time point onwards compared to control. Values represent the mean ± S.E.M, *p<0.05, **p<0.01, ***p<0.001, n=4 independent experiments each performed in triplicate.

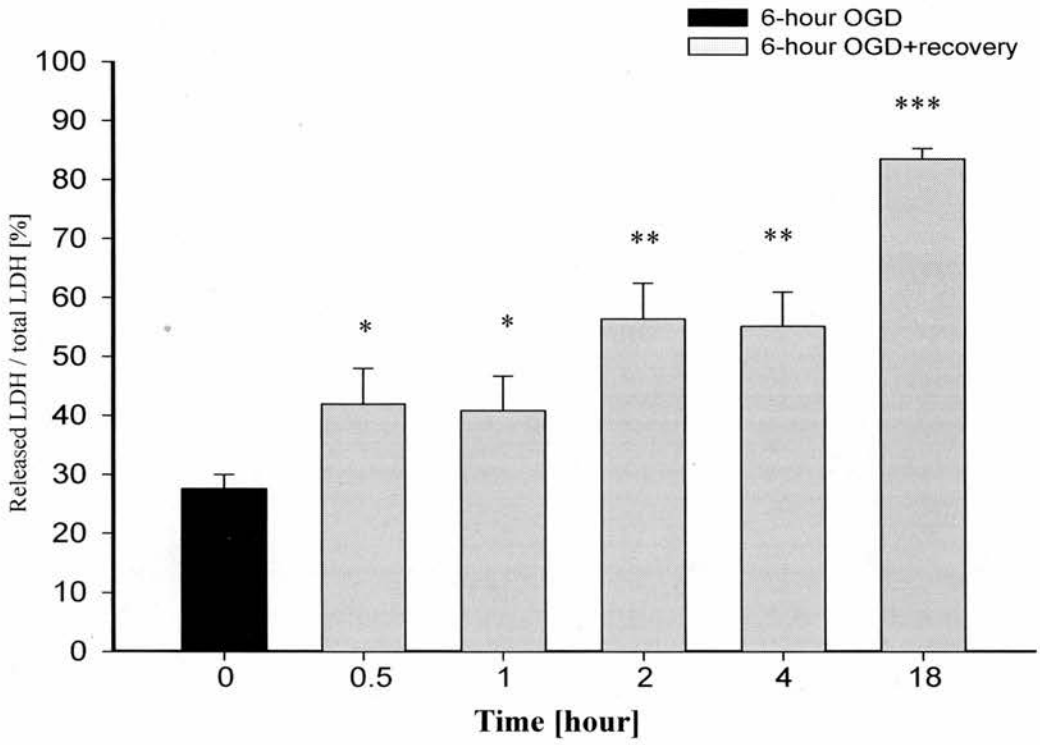


Fig 4-3. Effect of recovery following 6-hour OGD on the extent of LDH release. A 30-minute recovery period under normal conditions caused a significant increase in LDH release compared to the non-recovery control and this was further increased with longer recovery time. Values represent the mean ± S.E.M, *$p<0.05$, **$p<0.01$, ***$p<0.001$. n=4 independent experiments, each performed in triplicate.

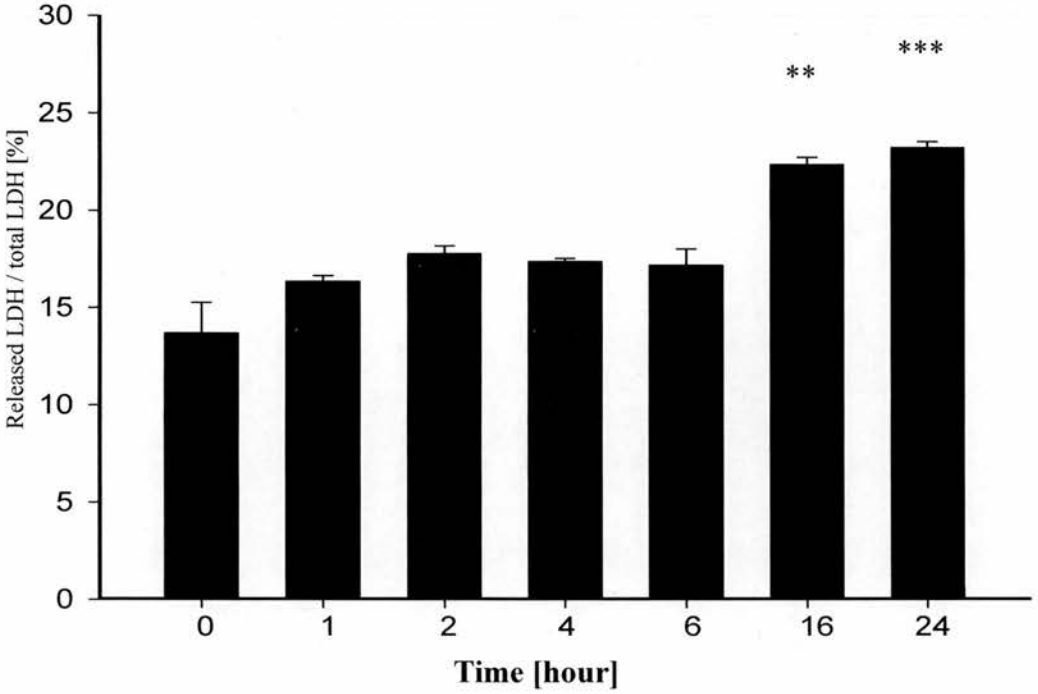


Fig 4-4. Time-course for LDH release induced by OGD&hypothermia. There was no significant difference in LDH release between control group and any time point of OGD&hypothermia exposure by 6 hours. However, LDH release was significantly increased after 16-hour and 24-hour exposure to OGD&hypothermia compared to control group. Values represent the mean ± S.E.M, **p<0.01, ***p<0.001. n=4 independent experiments, each performed in triplicate

4.2.2 Effects of cellular stressors on MR variant mRNA levels in primary cortical culture

RPA was used to detect the abundance of the three specific MR mRNA variants. Specific riboprobes were targeted to each individual MR variant exon 1 and complementary to the first 180 nucleotides of exon 2 (common to all MR mRNA variants). This overlapped incorporation of exon 2 enabled total MR mRNA to be measured simultaneously. Typically as shown in Fig 4-5 each sample contained a band representing the individual MR variant (MRα expected protected fragment size = 400nt; MRβ = 206nt; MRγ = 300nt) and a band representing the exon 2 overlap of MR (expected protected fragment size 180nt). The abundance of each protected fragment was quantified by densitometric analysis (Fig 4-5) and the absolute amount of total MR was calculated from the sum of the individual variant and the exon 2 band. The house-keeping gene β-actin served as the internal control (fragment size = 120nt). The transcript levels of each MR variant and total MR were expressed relative to β-actin to assess the absolute difference between treatment and vehicle group.

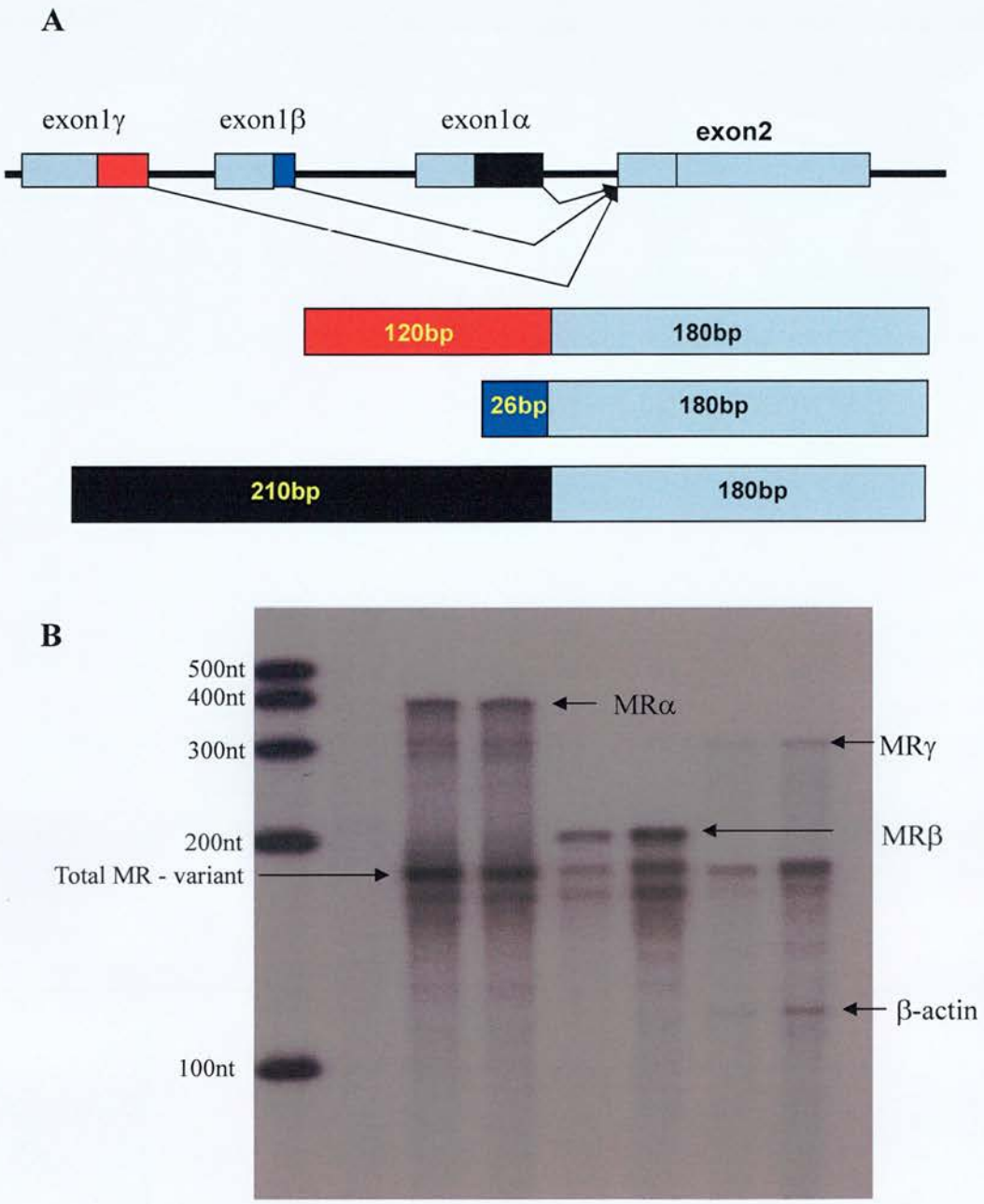


Fig 4-5. Schematic illustration of each riboprobe designed to target individual MR variant and total MR(A) and a representative autoradiographic image of an RPA gel in a pilot experiment (B). The RNA samples were extracted from rat primary cortical culture and performed in duplicate for individual riboprobe.

4.2.2.1 Proportion of MR variant in total MR in primary cortical culture

Under normal conditions, all three MR mRNA variants were detected in primary cortical culture by RPA with the MRβ the predominant MR variant while MRα and MRγ were expressed to a lower extent. The proportion of each individual MR variant calculated from all experiments showed MRβ to be the most highly expressed making up 56.55%±1.57% of the total amount of MR (α, β and γ), followed by MRγ at 20.15%±2.35% and MRα at 15.24%±0.8%.

4.2.2.2 Effect of cellular stressors on MR variants

Results showed that none of the three MR transcript levels were significantly altered in response to 10nM staurosporine (Fig 4-6A), 6-hour OGD (Fig 4-7A) or 6-hour hypothermia (Fig 4-8A) compared to the control group. However, 6-hour OGD&hypothermia significantly increased MRβ expression by 56%±8.8% ($p<0.05$), whilst having no effect on either MRα or MRγ (Fig 4-9B).

4.2.2.3 Effects of cellular stressors on total MR expression

Although 6-hour OGD&hypothermia significantly increased MRβ transcript level, total MR was not altered in response to this cellular stressor ($p=0.07$) (Fig 4-9C). Exposure to the other three cellular stressors staurosporine(Fig4-6B), 6-hour OGD (Fig 4-7B) or 6-hour hypothermia (Fig 4-8B) did not cause a significant increase in total MR expression compared to control group either.

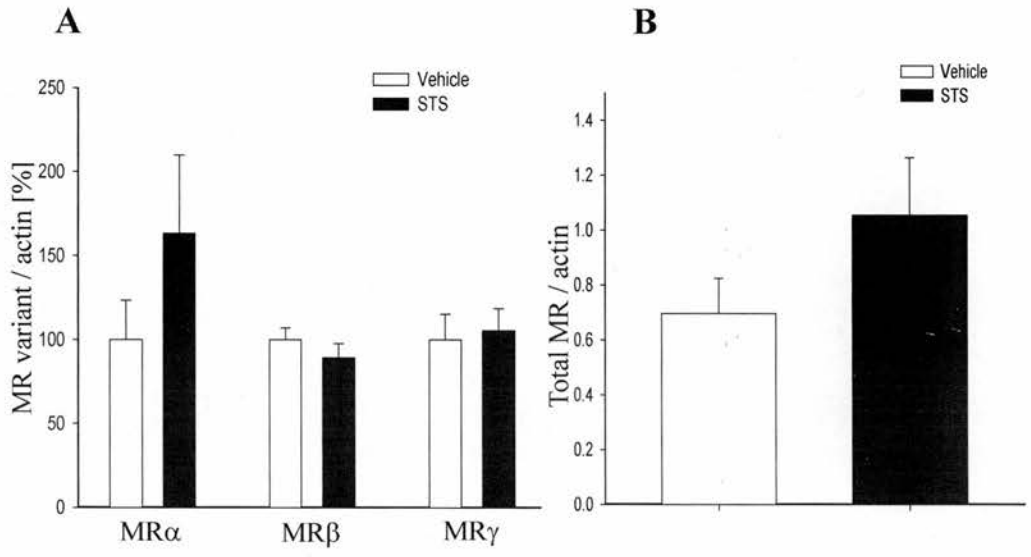


Fig 4-6. Quantitation of the effect of staurosporine on the expression of MR variants in primary cortical culture. The staurosporine-treatment group was expressed as a percentage of the vehicle group after normalization by internal control β-actin (A). The effect of staurosporine on total MR expression was also measured (B). 10nM staurosporine did not alter the expression of either MR variants or total MR. Values represent the mean ± S.E.M; n=6-9 independent experiments, each performed in triplicate.

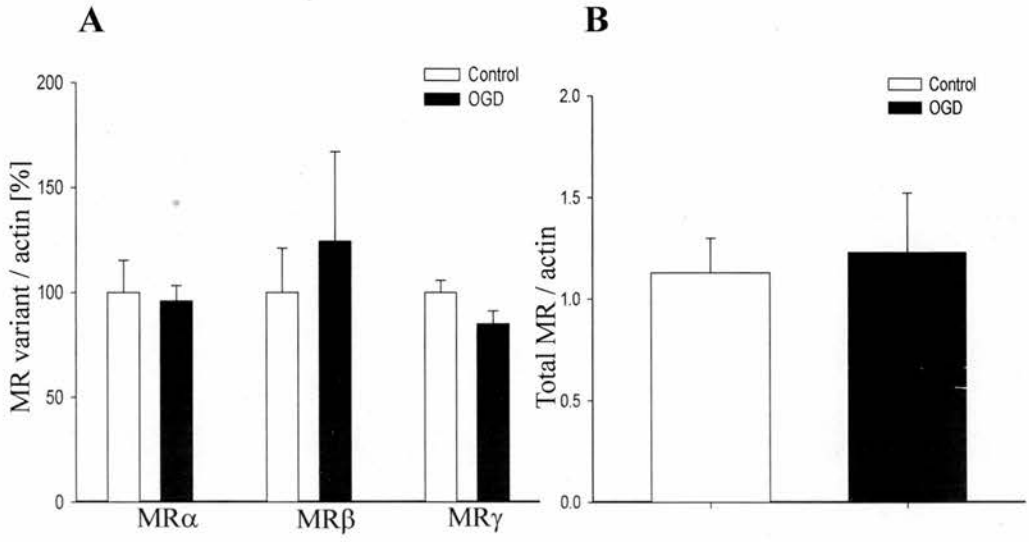


Fig 4-7. Quantitation of the effect of 6-hour OGD on the expression of MR variants in primary cortical culture. The OGD-treatment group was expressed as a percentage of the control group after normalization by internal control β-actin(A). The effect of OGD on total MR expression was also measured (B). 6-hour exposure to OGD did not alter the expression of either MR variants or total MR. Values represent the mean ± S.E.M, n=4-7 independent experiments, each performed in triplicate.

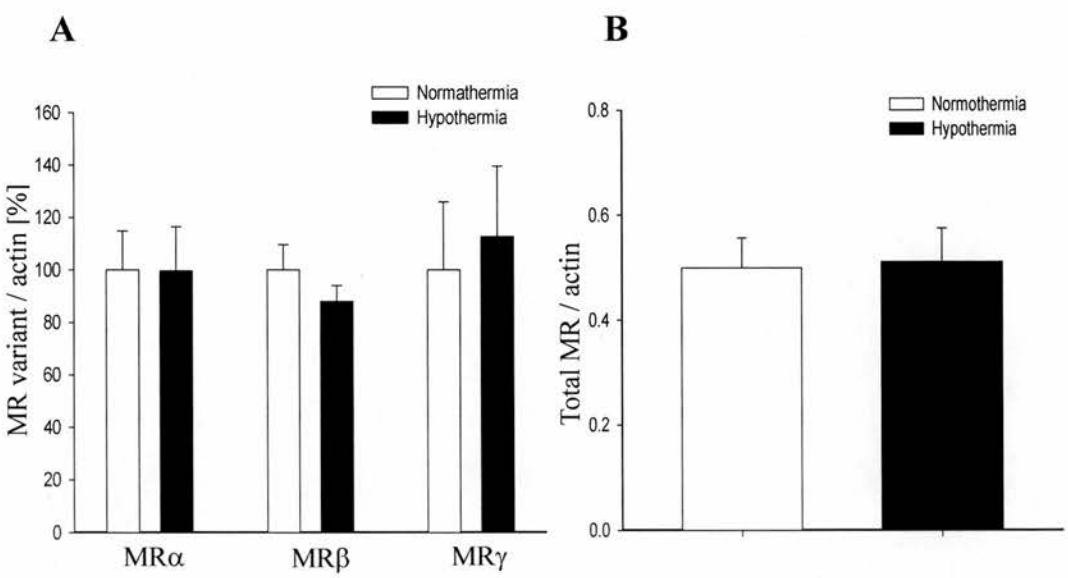


Fig 4-8. Quantitation of the effect of 6-hour hypothermia on the expression of MR variants in primary cortical culture. The hypothermia treatment group was expressed as a percentage of the normothermia group after normalization by β-actin(A). The effect of hypothermia on total MR expression was also measured(B). 6-hour exposure to hypothermia did not alter the expression of either MR variants or total MR. Values represent the mean ± S.E.M, n=4-6 independent experiments, each performed in triplicate.

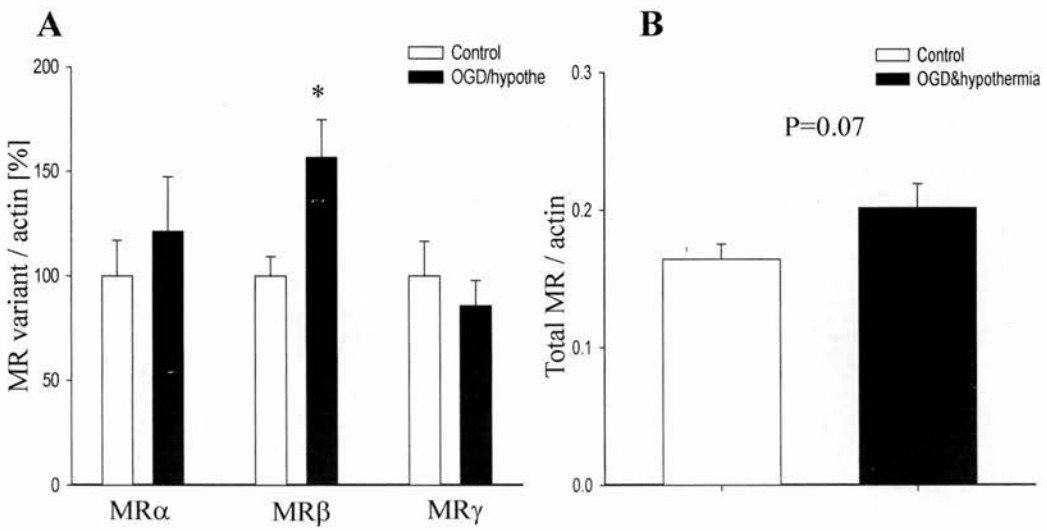


Fig 4-9. Quantitation of the effect of 6-hour OGD&hypothermia on the expression of MR variants in primary cortical culture. The treatment group was expressed as a percentage of the control group after normalization by internal control β-actin(A). The effect of OGD&hypothermia on total MR expression was also measured (B). 6-hour OGD&hypothermia significantly increased MRβ variant expression ($p<0.05$) while had no effects on MRα or MRγ. Total MR showed a tread to be increased in response to 6-hour OGD&hypothermia ($p=0.07$). Values represent the mean ± S.E.M, *$p<0.05$, n=6 independent experiments, each performed in triplicate.

4.3 Discussion

In this chapter, the effects of different types of cell stress on the transcript levels of each MR variant and of total MR were investigated in the primary cortical culture. Findings are summarized in Tab 4-1.

Table 4-1 Summary of transcript levels of MRα, MRβ and MRγ in response to different types of cellular stress in primary cortical culture

	MRα	MRβ	MRγ	Total MR
Staurosporine	↔	↔	↔	↔
OGD	↔	↔	↔	↔
Hypothermia	↔	↔	↔	↔
OGD&hypothermia	↔	↑	↔	↔
↔ no change; ↑ significant increase				

The data in chapter 3 provided a clue that both MRα and MRβ transcripts can be potentially induced in response to OGD&hypothermia, since their promoter activities were significantly increased in differentiated PC12 cells. However, measurement of transcript levels in primary cortical culture after 6-hour OGD&hypothermia showed only expression of MRβ but not MRα was augmented, whilst other stressors had no effects on any of the MR variants. Clearly, this demonstrates and further verifies that (i) there is indeed a differential induction of MR variant mRNA expression. (ii) this induction is confined specifically to OGD&hypothermia.

Hypothermia is known to be neuroprotective, (Busto *et al.* 1989; Buchan *et al.* 1990; Baker *et al.* 1995), and this was verified in this system when measuring the extent of cell damage (LDH release) in primary cultures exposed to OGD and OGD&hypothermia (4.2.1.2 OGD&hypothermia). There was no measurable

change in LDH release in OGD&hypothermia treated cells over several time points up to 6 hours compared to controls whereas OGD treated cells showed an increase in LDH release over time, indicting that hypothermia exerts a protective effect against OGD-induced damage. Although this experiment was performed in an *in vitro* primary culture, the induction of MRβ strongly indicated that it is the primary sensor involved in such an event. This may be due to the intrinsic property of MRβ, which has been already indicated to be involved in neurodevelopment in the embryonic hippocampus (Vazquez *et al.* 1998). Given the notion that the brain injury often triggers the cellular activities recapitulating the events that underlie development (Chen *et al.* 2005), it is plausible that the induction of MRβ is predominantly responsive to injury. Furthermore, MRβ was a principle variant in total MR in primary cortical culture occupying over 50% MR mRNA. This was partially in agreement with previous findings that this variant was highly expressed in the developing hippocampus (Vazquez *et al.* 1998). Taken together, MRβ is an essential variant which acts as the primary transcript responsive to injury through the activation of its own promoter. This discovery sheds light on the development of an MR-based neuroprotective drug specifically in neurons where MRβ can act as a potential target.

The expression of total MR was not affected by any of the cellular stressors in primary cortical culture. However, there was a trend that exposure to 6-hour OGD&hypothermia caused an increase in the expression of total MR ($p=0.07$). This was believed to be contributed by the increased MRβ transcript solely. The failure of induction of total MR may lie in the limited increased magnitude of MRβ, which was not enough to alter the total MR expression. Besides, the calculation of total MR by sum of individual MR variant and exon 2 appears to be an indirect method, which might also shields the true biological changes.

Although MRα appears to be less abundant in primary cortical cultures than in the adult brain *in vivo*, it is still an interesting target to explore. In Chapter 3, the promoter activity of MRα was significantly increased after exposure to OGD&hypothermia in differentiated PC12 cells, which implied a potential increase in transcript level. Surprisingly the mRNA level of MRα in primary cortical culture did not confirm such hypothesis. This discrepancy may be explained by the fact that RNA was extracted immediately after exposure to OGD&hypothermia. This may mask the potential change of MRα expression since rates of transcription vary between genes and the time at which the change in MRα expression may not have been captured. For the promoter experiments all analysis was performed after 24 hours to maximize luciferase synthesis and steady state levels. Secondly, the stability of MRα mRNA also needs to be considered (Mignone *et al.* 2002) particularly if its half life is less than 6 hours. A protocol which measures actual change in the rate of transcription of MR variant expression may elucidate if expression is altered by OGD&hypothermia.

It has been shown that 10nM staurosporine addition for 1 hour in primary hippocampal and cortical cultures caused an induction of MR abundance (Macleod *et al.* 2003). The promoter study showed MRα promoter activity was significantly increased by 10nM staurosporine regardless of PC12 cell phenotypes, making it a strong candidate responsible for the induction of MR. Surprisingly, staurosporine failed to induce the mRNA level in primary culture. Notably, the variables between experiments may be too big to reach a statistical significance, probably shielding the true biological effects of staurosporine. Therefore, it may be worth trying an alternative method such as real-time PCR or semi-quantitative PCR to measure the MRα expression directly.

The results form both chapter 3 and 4 demonstrate that the MRγ variant is not

affected by any of the cellular stressors *in vitro*, either at the promoter or mRNA level. In primary cortical culture, it occupied approximately 20% of total mRNA, even higher than MRα, but what its cellular role is remains to be elucidated.

In this study, recovery was excluded following OGD, hypothermia or OGD&hypothermia in contrast to the promoter activity experiments in Chapter 3 due to high proportion of cell damage even for a minimum period. It is known that restoration of circulation in global cerebral ischaemia leads to delayed neuronal death in some vulnerable regions in the brain which may occur hours to days after the insult (Pulsinelli *et al.* 1979; Kirino 1982; Pulsinelli *et al.* 1982). Although increased OGD&hypothermia in primary cortical culture, the induction of MRβ could be prompt that only lasted a few hours and disappeared quickly when the recovery was restored. Alternatively, the other two MR variants MRα and MRγ may also mount a late induction.

4.4 Concluding marks

This chapter provides evidence for specific transcriptional regulation of the MR gene by OGD&hypothermia in primary cortical cultures. The data shows that OGD&hypothermia significantly increases the MRβ variant and demonstrates a novel role for MRβ in the response to injury. This suggests that targeting MRβ in neurons may be a way of treating neuronal injury. However, these observations are based on *in vitro* experiments and to be of biological or clinical relevance, this phenomenon needs to be verified in an *in vivo* system.

Chapter 5

MESSENGER RNA LEVELS OF THE MINERALOCORTICOID RECEPTOR VARIANTS IN HYPOTHERMIC ANOXIA NEONATAL RAT

5.1 Introduction

The work in chapter 3 and chapter 4 has identified that MRβ variant is the primary sensor responsive to the cellular injury. Although the *in vitro* studies provide a good initial screen for the differential regulation of MR, there are still several limitations in particular they do not reflect the actual physiological environment where the neurons reside and exert their functions. For example, substantial less glia cells are present in primary cortical culture in comparison to the brain *in vivo* and the architecture of the cerebral cortex is completely disrupted that may cause altered intracellular communication and activities. More crucially, it is not known whether the cell culture itself has an impact on the gene expression in the cells because the change of the environment will certainly be considered as a "stressful" event that might disrupt the cellular homeostasis and subsequently lead to a series of cellular responses. Therefore, to validate the changes in the expression profiles of the MR variant mRNA transcripts *in vitro* and of biological importance, this chapter will examine the transcription regulation of MR in *in vivo* models. Unfortunately the adult rat hypothermic global ischaemia model where previous work was carried out is not available, the work was carried out in a well characterized cerebral anoxic neonatal rat model where the body temperature can be easily manipulated (Caputa *et al.* 2001).

Cerebral hypoxia remains a major health problem for infant and children that can induce chronic neurological dysfunction throughout their lifespan, including impaired learning and memory, cerebral palsy, mental retardation and epilepsy (Buwalda *et al.* 1995; Vannucci *et al.* 1997; Fan *et al.* 2005). It is defined as partial or complete lack of oxygen supply to the brain due to obstructed airways or cut-off of the atmospheric oxygen. Severe hypoxia is referred to as anoxia which can cause irreversible pathological changes and neuronal loss in brain regions particularly those vulnerable to oxygen shortage such as the hippocampus (Nyakas *et al.* 1996; Oechmichen *et al.* 2006). At the cellular level, anoxia shares the similar pathophysiological process with cerebral ischaemia, including ion disturbances, Ca^{2+} overload, formation of oxidative free radicals and apoptosis (Vannucci 1990; Johnston *et al.* 2001). In addition, a variety of studies showed the endogenous survival factors are also mounted in this type of animal models (Vexler *et al.* 2001).

In hypothermic anoxia neonatal rat models, changes in the expression of MR variant transcripts were measured specifically in the hippocampus since (i)MR is predominantly expressed in this part of the brain and (ii)the hippocampus is particular vulnerable to the damaging effects of ischaemia. The aims of this chapter were specifically to investigate:

(i) If MR gene expression is induced in hypothermic anoxic neonatal rats.

(ii) Which MR variant(s) is/are responsible for the induction of total MR in this animal model.

5.2 Results

The abundance of total MR was measured by *in situ* hybridization using 513nt

cRNA riboprobe targeting the 3'-untranslated region which was reported in previous study (Yau *et al.* 1994). For the detection of individual MR variant, another three cRNA riboprobes were synthesized and specifically complementary to the entire MRα, MRβ and MRγ exon1 region, thereby ensuring the specificity for each receptor variant. Quantification was carried out using silver grain counts, which is expressed as the ratio of total grey levels relative to number of radioactive hybrids per neuron.

5.2.1 Basal distribution pattern in hippocampus

Grain counting analysis showed that under normal conditions, total MR was most enriched in CA3 subfield while least abundant in CA4. The distribution of total MR expression in hippocampal subfields from the highest level was CA3>DG>CA2>CA1>CA4. The pattern was same as for the MR variants, only with the exception that MRβ and MRγ had a higher expression in CA1 than CA2 (Tab 5-1).

5.2.2 Effect of hypothermic anoxia

As shown in Fig 5-2, in comparison to sham group, anoxia at normothermia did not alter MR mRNA in any of hippocampal subfields by one-way ANOVA analysis. However, anoxia&hypothermia caused a significant increase in hippocampal MR expression by 119.5% in CA1 ($p<0.05$), 46.68% in CA2, ($p<0.05$), 48.36% in CA3 ($p<0.05$), 89.24% in CA4 ($p<0.001$) and 46.91% in DG ($p<0.05$). In addition, hypothermia alone was also associated with increased MR mRNA expression in all hippocampal subfields except CA2 ($p=0.052$).

Subsequently, the transcript level of each of MR variants was tested using the

specific cRNA riboprobe. Neither MRα (Fig 5-4) nor MRγ (Fig 5-8) mRNA levels were significantly altered in response to any of the treatments in any of the hippocampal subfields.

However, anoxia&hypothermia significantly increased MRβ mRNA levels in comparison to the sham group, by 54.78% in CA1 ($p<0.05$), 66.48% in CA2 ($p<0.05$), 42.56% in CA3 ($p<0.01$), 51.47% in CA4 ($p<0.05$) and 60.14% in DG ($p=0.001$). The induction of MRβ was also found in response to hypothermia alone in hippocampal CA1 (by 128.6%, $p<0.05$), CA2 (by 51%, $p<0.05$), CA3 (by 66.67%, $p<0.05$), CA4 (by 74.06%, $p<0.01$) and DG (by 69.5%, $p<0.05$) compared to the sham group. However, anoxia alone did not affect the expression of MRβ mRNA abundance in the hippocampus (Fig 5-6). This suggests the induction of total MR expression was solely contributed by MRβ.

5.2.3 Two-way ANOVA

A two-way ANOVA considering two factors of hypothermia and anoxia for each cRNA probe versus the sham group was performed to evaluate their impact on the transcript levels of each MR variant and total MR. The effect of hypothermia alone was statistically significance in MRβ (F 21.591; $p<0.001$) and total MR (F 5.696; $p<0.05$), but anoxia did not affect either of them (MRβ: F 0.23, $p=0.63$; total MR: F 0.34, $p=0.56$). The interaction between hypothermia and anoxia did not reach statistical significance for MRβ (F 0.676; $p=0.413$) or total MR (F 1.889; $p=0.172$), suggesting the induction of MRβ was purely rely on the hypothermia. In addition, neither MRα nor MRγ was affected by these two factors.

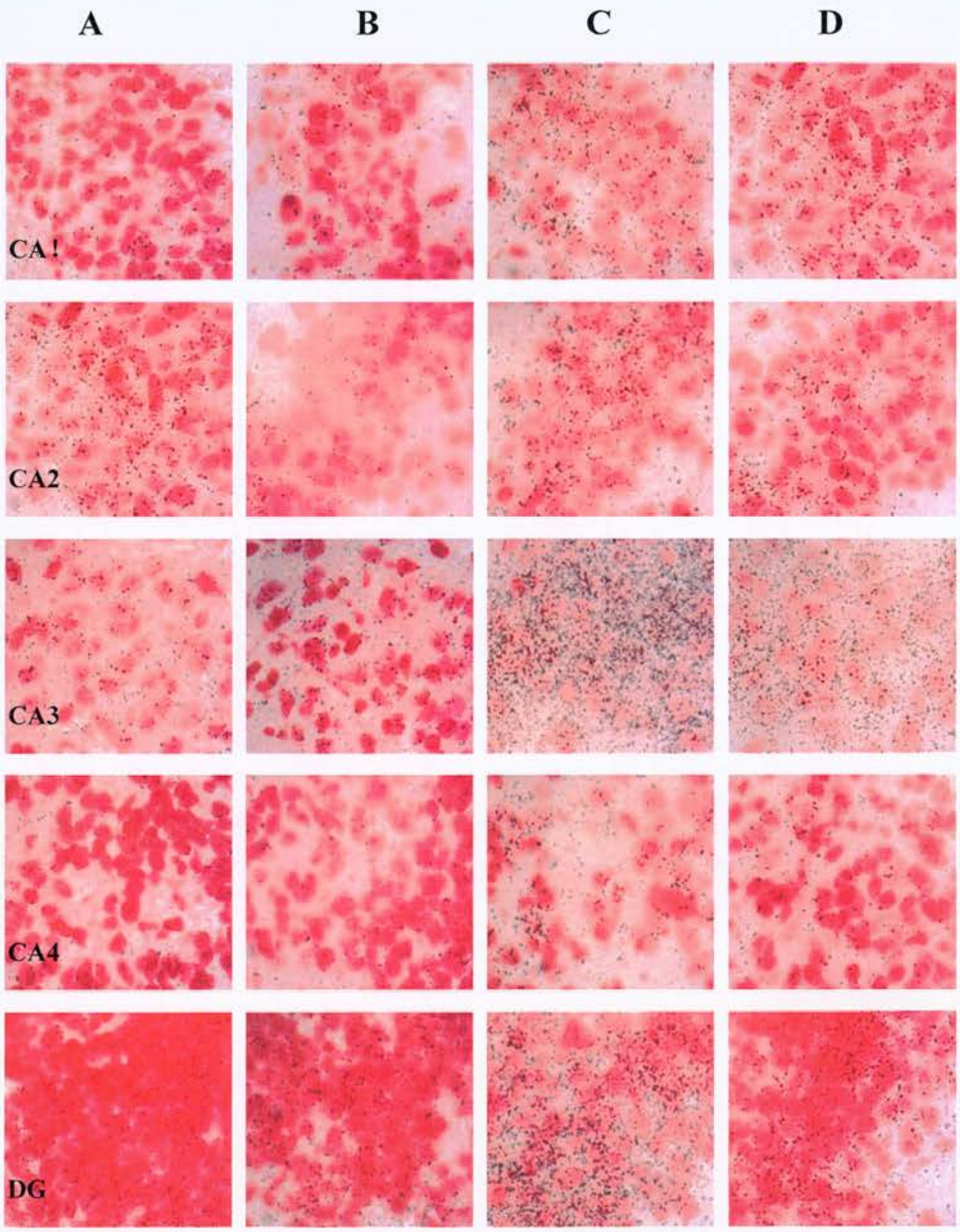


Fig 5-1. Representative pictures for total MR mRNA levels in hypothermic anoxia neonatal rats. [Column (A)]: sham; [Column (B)]: Anoxia; [Column (C)]: Hypothermia alone; [Column (D)]: anoxia&hypothermia

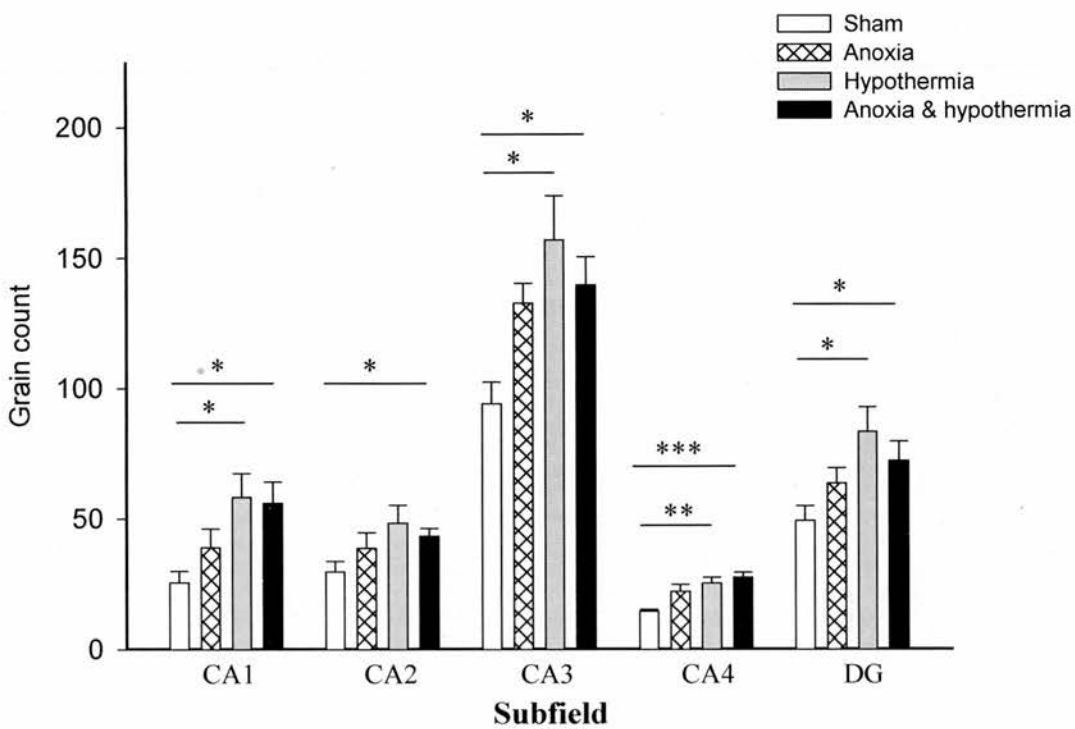


Fig 5-2. Effect of hypothermic anoxia on MR expression in neonatal rats. MR expression was significantly increased in all of hippocampal subfields by anoxia&hypothermia. Hypothermia alone also induced an increase in MR expression in hippocampal CA1, CA3, CA4 and DG subfields. Data represent mean ± S.E.M, *p<0.05; **p<0.01; ***p<0.001, n=6 animals per group with each performed in triplicate brain sections.

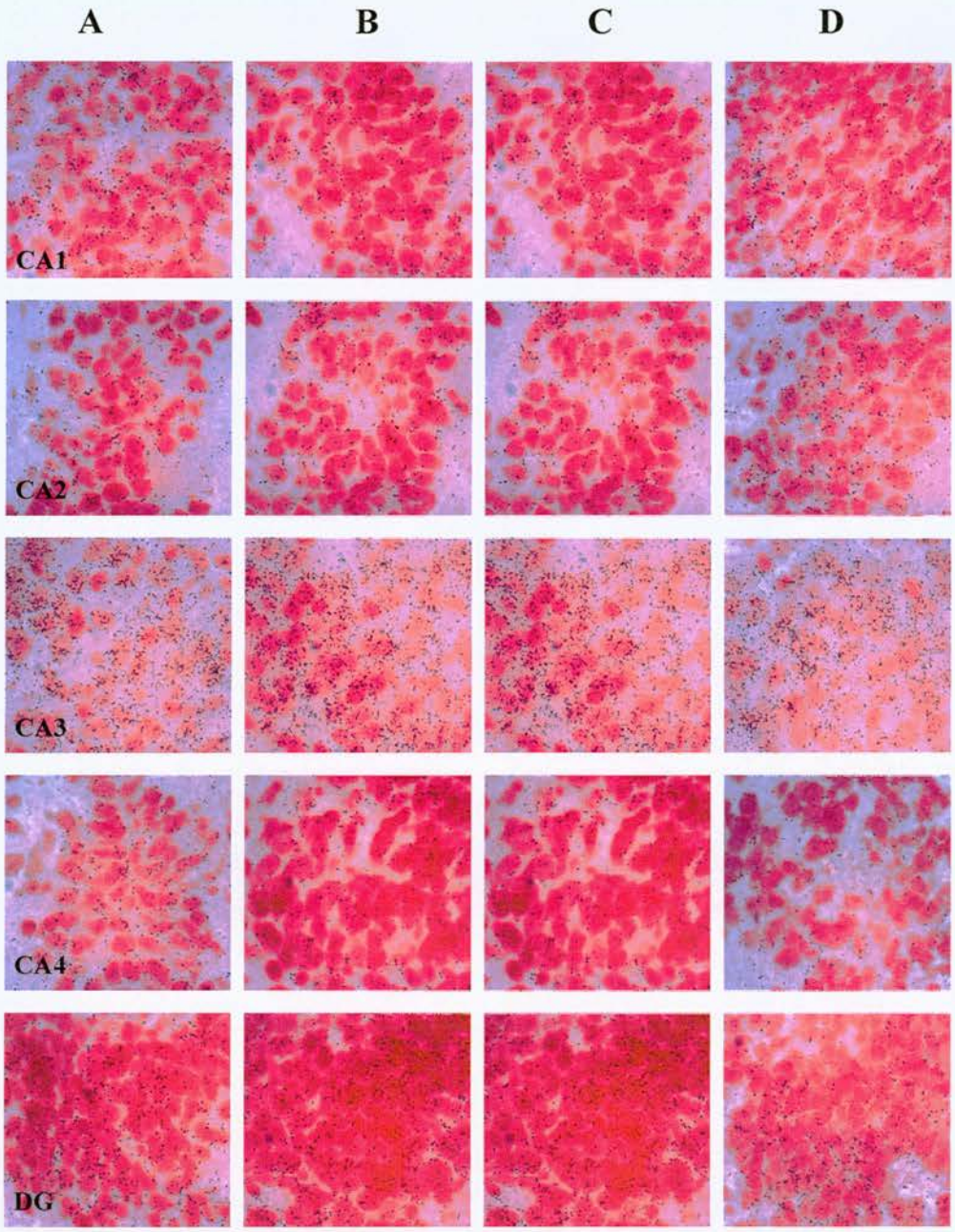


Fig 5-3. Representative pictures for MRα mRNA levels in hypothermic anoxia neonatal rats. [Column (A)]: Sham; [Column (B)]: Anoxia; [Column (C)]: Hypothermia alone; [Column (D)]: Anoxia&hypothermia

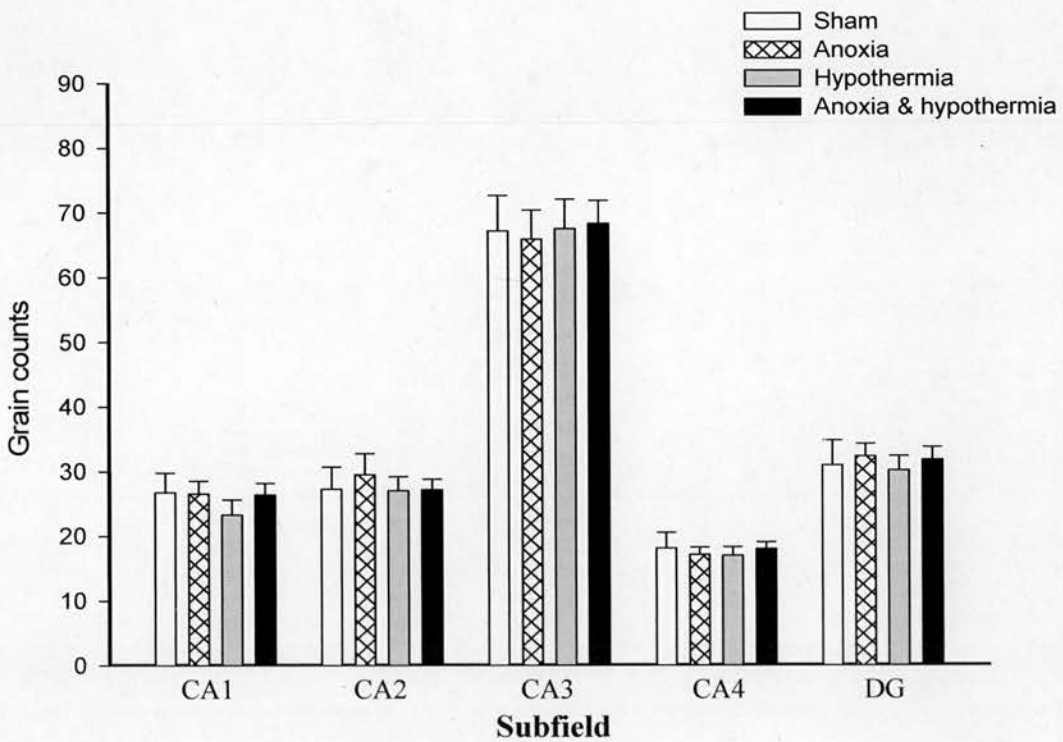


Fig 5-4. Effect of hypothermic anoxia on MRα expression in neonatal rats. There was no significant difference in MRα expression between treatment group and sham group in any of the hippocampal subfields. Data represent mean ± S.E.M, n=6 animals per group with each performed in triplicate brain sections.

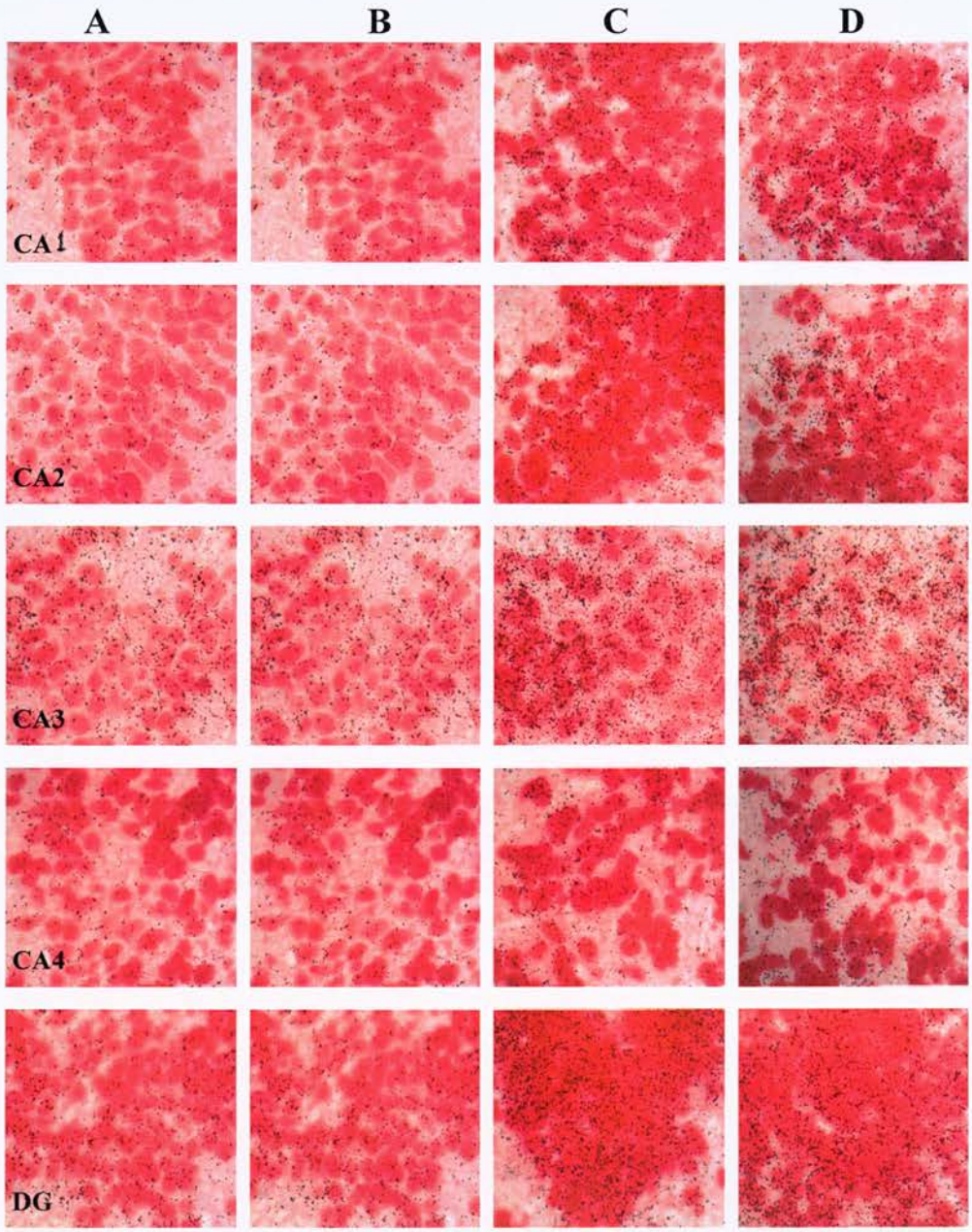


Fig 5-5. Representative pictures for MRβ mRNA levels in hypothermic anoxia neonatal rats. [Column (A)]: sham; [Column (B)]: Anoxia; [Column (C)]: Hypothermia alone; [Column (D)]: Anoxia&hypothermia

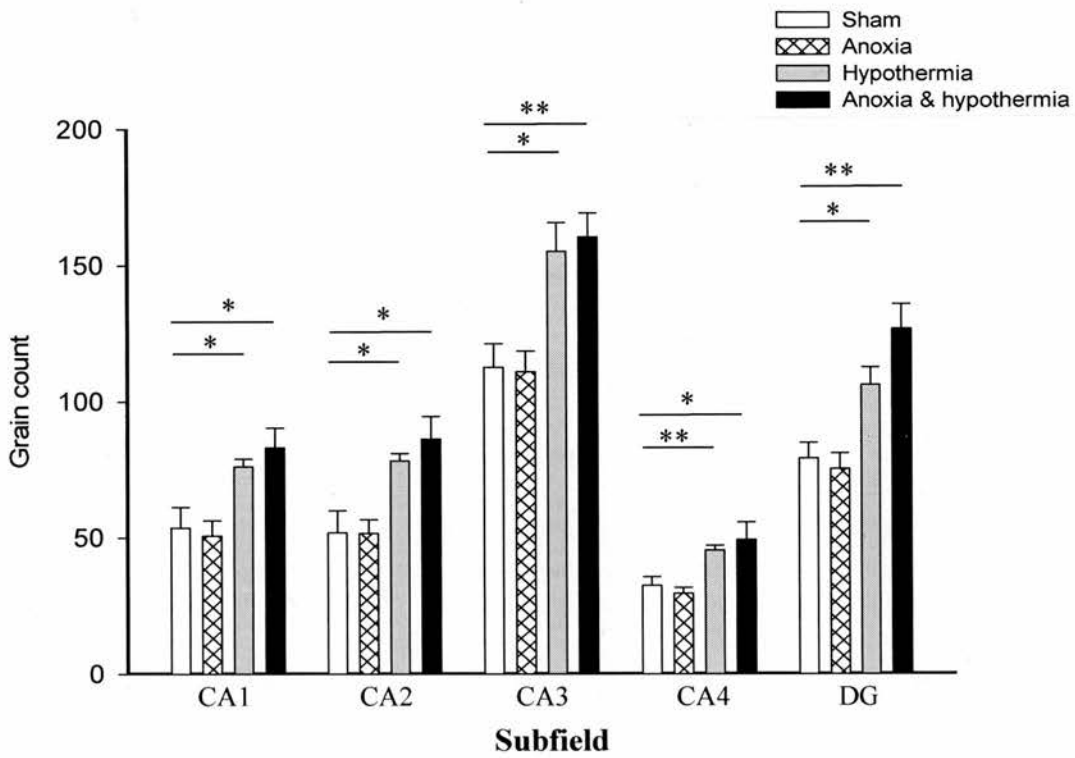


Fig 5-6. Effect of hypothermic anoxia on MRβ expression in neonatal rats. MRβ expression was significantly increased in all of hippcoampal subfields in response to both hypothermia and anoxia&hypothermia compared to sham group. Data represent mean ± S.E.M, *$p<0.05$; **$p<0.01$; n=6 animals per group with each performed in triplicate brain sections.

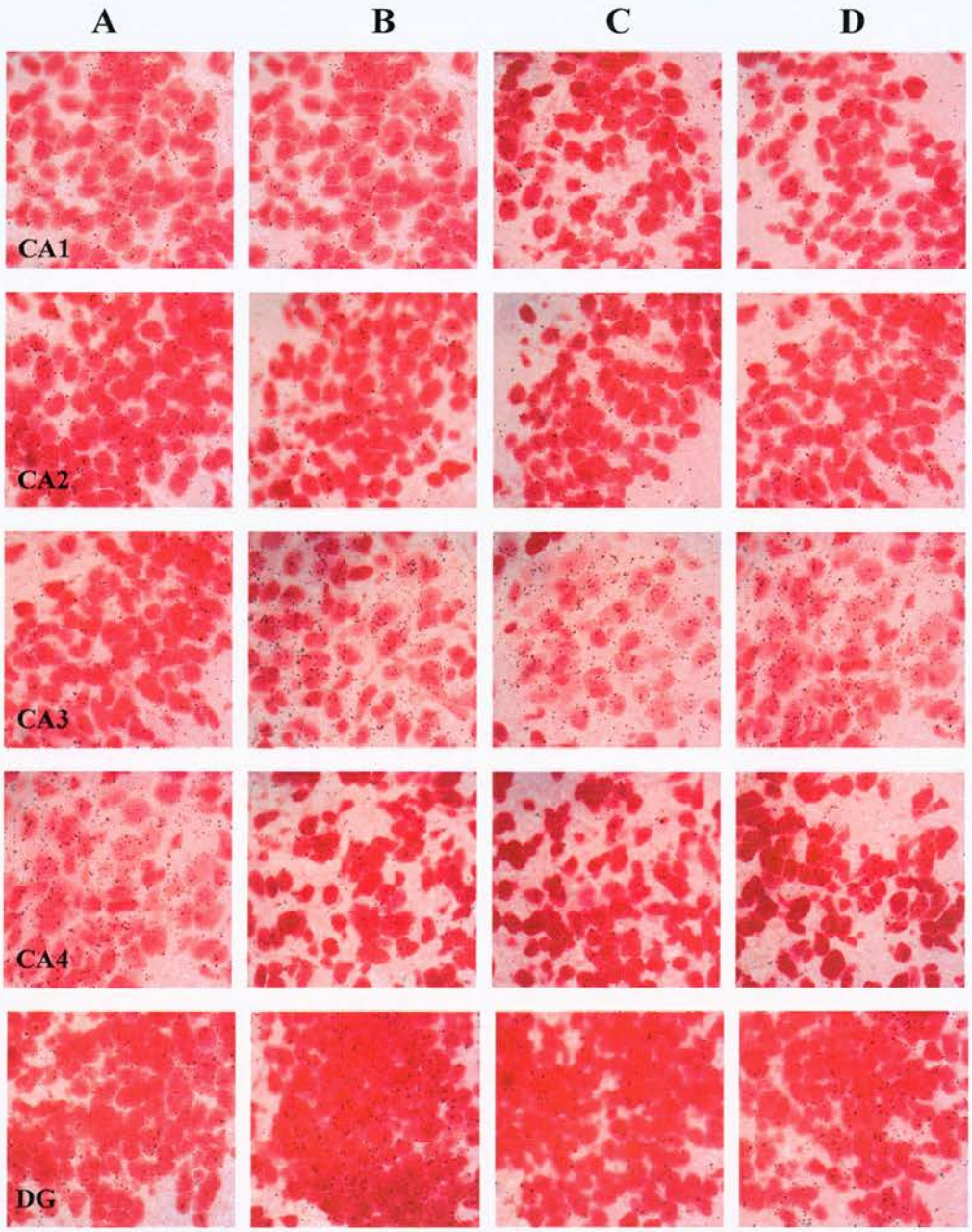


Fig 5-7. Representative pictures for MRγ mRNA levels in hypothermic anoxia neonatal rats. [Column (A)]: sham; [Column (B)]: Anoxia; [Column (C)]: Hypothermia alone; [Column (D)]: Anoxia&hypothermia

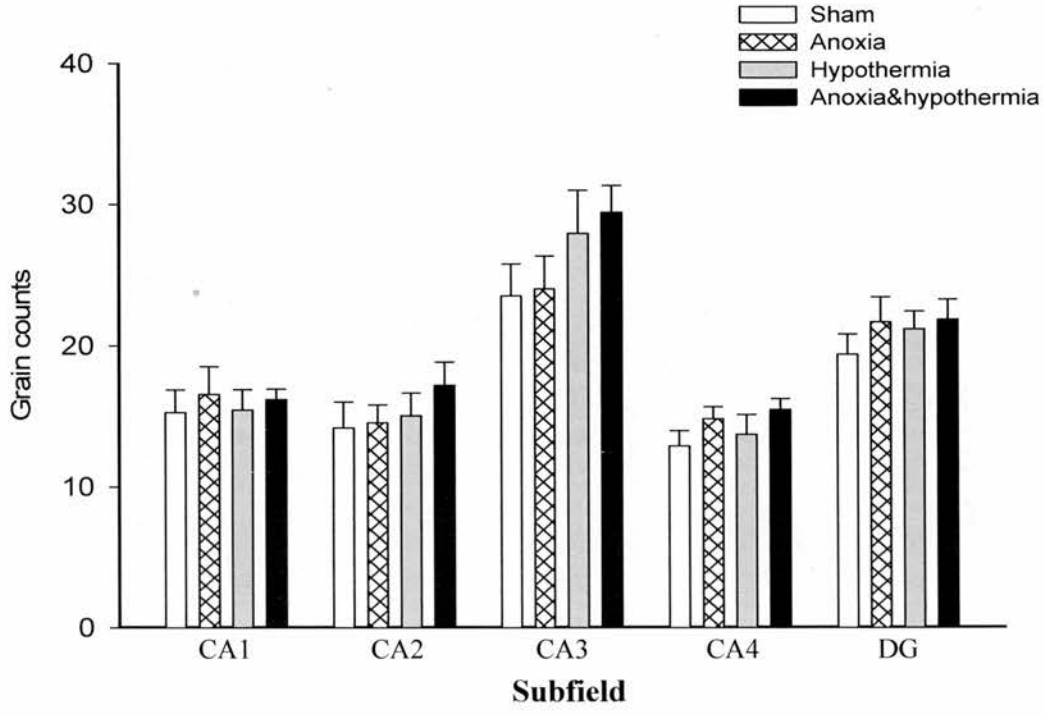


Fig 5-8. Effect of hypothermic anoxia on MRγ expression in neonatal rats. There was no significant difference in MRγ expression between treatment group and sham group in any of the hippocampal subfields. Data represent mean ± S.E.M, n=6 animals per group with each performed in triplicate brain sections.

Table 5-1 Effect of anoxia and hypothermia on expression of MR and variants in neonatal rat

	Control		Anoxia	
MR	Normothermia	Hypothermia	Normothermia	Hypothermia
CA1	25.49 ± 4.36	58.27 ± 9.17[a]	38.99 ± 7.16	55.95 ± 8.26[a]
CA2	29.61 ± 4.1	48.43 ± 6.85	38.64 ± 6.11	43.43 ± 2.82[a]
CA3	94.23 ± 8.23	157.05 ± 16.87[a]	132.74 ± 7.66	139.74 ± 10.81[b]
CA4	14.51 ± 0.72	25.26 ± 2.2[b]	22.03 ± 2.72	27.46 ± 1.89[c]
DG	49.19 ± 5.73	83.38 ± 9.53[a]	63.57 ± 5.87	72.27 ± 7.38[a]
MRα				
CA1	26.75 ± 3.03	23.3 ± 2.31	26.59 ± 1.97	26.44 ± 1.74
CA2	27.32 ± 3.39	27.08 ± 2.17	29.56 ± 3.22	27.21 ± 1.60
CA3	67.21 ± 5.47	67.53 ± 4.54	67.94 ± 4.50	68.33 ± 3.56
CA4	18.14 ± 2.42	16.98 ± 1.35	17.14 ± 1.15	18.02 ± 1.04
DG	30.97 ± 3.88	30.20 ± 2.12	32.34 ± 1.93	31.81 ± 1.99
MRβ				
CA1	53.68 ± 7.57	76.11 ± 2.82[a]	50.71 ± 5.58	83.08 ± 7.28[a]
CA2	51.83 ± 8.22	78.27 ± 2.77[a]	51.61 ± 5.05	86.29 ± 8.34[a]
CA3	112.64 ± 8.65	155.25 ± 10.52[a]	111.02 ± 7.59	160.58 ± 8.74[b]
CA4	32.56 ± 3.12	45.44 ± 1.73[b]	29.55 ± 2.22	49.32 ± 6.44[a]
DG	79.18 ± 5.77	106.18 ± 6.44[a]	75.38 ± 5.75	126.79 ± 9.14[b]
MRγ				
CA1	15.27 ± 1.61	15.45 ± 1.44	16.55 ± 1.97	16.18 ± 0.75
CA2	14.17 ± 1.83	15.04 ± 1.60	14.53 ± 1.27	17.19 ± 1.67
CA3	23.51 ± 2.78	27.95 ± 3.05	24.01 ± 2.34	29.45 ± 1.90
CA4	12.88 ± 1.10	13.72 ± 1.39	14.82 ± 0.85	15.47 ± 0.77
DG	19.37 ± 1.44	21.18 ± 1.26	21.67 ± 1.76	21.85 ± 1.41

[a]$P<0.05$ vs normothermia control; [b]$P<0.01$ vs normothermia control; [c]$P<0.001$ vs normothermia control. One-way ANOVA *post hoc* test.

5.3 Discussion

The data in this chapter demonstrated that MR gene expression was differentially regulated in hypothermic anoxic neonatal rats *in vivo*. This was the last model system used in this project and the results were summarized in Tab 5-1.

In chapter 4, only MRβ mRNA level was significantly increased in response to OGD&hypothermia in primary cortical culture but failed to induce an increase in total MR expression. This *in vitro* study suggested that MRβ was the primary spliced variant involved in hypothermic neuroprotection following ischaemic insult. However, the question remains on whether this is of biological relevance in *in vivo* animal models. The results in this chapter confirmed the findings in chapter 4, that MRβ was indeed the sole variant responsible for the induction of total MR in response to hypothermic anoxia, establishing the important role of MRβ in mediating MR-based neuroprotection. In adult rats, increased MR gene expression was limited to CA3 and CA4 subfields by hypothermic transient global ischaemia (Macleod *et al.* 2003), whereas the induction of MR was found in all hippocampal subfields in neonatal rat treated with hypothermic anoxia. The difference was probably attributable to the different transcript levels of MRβ between neonatal and adult rats. Vazquez reported MRβ was highly expressed in the developing hippocampus but declined in adults (Vazquez *et al.* 1998). In primary cortical culture, MRβ was a predominant variant occupying over 50% of total MR (4.2.2.1). Therefore, it is assumed that inadequate expression of MRβ in adult rats compared to newborn animals may not be able to induce an overall increase in MR abundance in response to hypothermic ischaemia in hippocampal CA1, CA2 or DG. This hypothesis can be validated by performing the same experiments to measure the expression of each MR variant in hypothermic ischaemic adult rats. In addition, this elicits an important clinical strategy to

develop the potential therapeutic interventions based on enhancing MR expression in which MRβ can be a primary target.

Surprisingly, two-way ANOVA considering the two factors, hypothermia and anoxia, indicated that hypothermia was a significant factor involved in the expression of total MR *in vivo*. *Post hoc* analysis demonstrated that hypothermia alone augmented the expression of total MR in hippocampal subfields except CA2. Currently there are very few reports available correlating MR expression to the temperature in the central nervous system. Sun demonstrated that the temperature at 26°C can significantly increase the mRNA and protein levels of MR in heart, kidneys and hypothalamus in mice compared to the normothermic condition (Sun *et al.* 2008). The mechanism of the induction in these tissues was assumed to be the role of MR in mediating blood pressure. At hypothalamus level, MR induction was independent from the circulating concentration of aldosterone, suggesting other mechanisms than autoregulation may direct the expression of MR at lower temperature (Sun *et al.* 2008). Coincidently, the data in this chapter demonstrated that hippocampal MR expression was also subjected to such thermal regulation in neonatal rat and this was believed to be mediated by MRβ variant solely. The mechanism by which MRβ is subjected to thermal regulation in the hippocampus has not been reported, but it might be related to the developing brain, since hypothermia treatment in adult rat did not change MR abundance in any of hippocampal subfields (Macleod *et al.* 2003). Another reason may be the less severity of the anoxic insult in this neonatal model which was not enough to mount an MR-mediated survival response. The brain of a newborn animal is believed to be more resistant to a hypoxic/anoxic insult in contrast to the adults (Cherubini *et al.* 1989; Haddad *et al.* 1990). A quick examination of stained neonatal brain sections post-anoxia revealed an absence of neuronal damage in contrast to the adult rat (Macleod *et al.* 2003). Previous studies have shown that

immature neurons demanded less energy input at the developmental stage i.e. lower levels of neurotransmitter synthesis and glucose utilization (Vannucci *et al.* 1989; Nehlig *et al.* 1993; Krajnc *et al.* 1994). Furthermore, the neonatal rats maintained their core temperature at 32-33°C under normal conditions (Bertin *et al.* 1993; Caputa *et al.* 2001), which is the hypothermic condition for the adult rat and presumed to protect the neonatal developing neurons from anoxic insult. This can be partially confirmed by the study using hyperthermia at 37°C and 39°C, which significantly reduced the tolerance of neonatal rats in exposure to anoxia (Rogalska *et al.* 2005). Therefore, lowered core temperature is beneficial for resistance to anoxic insult.

In addition, it is note worth to mentioning GR may also play a fundamental role in the induction of MR. Hypothermia was associated with reduced GR mRNA levels compared to normothermic ischaemia in adult rats (Macleod *et al.* 2003), suggesting a potential role for modulation of steroid receptors in response to ischaemia. Gradually increased circulating steroid concentration would have a greater impact on potentially neurotoxic GR signalling than on MR signalling, due to relative lower affinity of steroids for GR (Reul *et al.* 1985). Hence, it is assumed that increased MR may have evolved as a mechanism to enhance the MR signalling to counteract GR-induced deteriorating effects (Macleod *et al.* 2003). Our preliminary data in the same neonatal rat models using GR-specific riboprobe demonstrated that (i) the transcript of GR was detectable in hippocampal CA1 and CA2 but extremely low in the other three subfields (CA3, CA4 and DG) and (ii) GR expression was not significantly altered by either hypothermia or anoxia&hypothermia in comparison to sham groups in CA1 or CA2 subfield (Appendix 5). This rules out the possibility that the increased MR expression was attributable to decreased GR and suggests the up-regulation of MR in hypothermic anoxic neonatal rat may be subjected to injury-specific mechanisms irrespective of GR.

MRα variant was consistently expressed in the hippocampus from birth to adult (Vazquez *et al.* 1998) and was responsive to corticosterone at promoter and transcript levels in neurons (Castren *et al.* 1995). Although the promoter activity was augmented by staurosporine and OGD&hypothermia, the transcript level of MRα was not altered in either primary cortical culture *in vitro* or neonatal rats *in vivo*, indicating this is not a primary sensor involved in the induction of MR.

Although MRγ is important in the neonatal rat with considerable expression in developing hippocampus (Vazquez *et al.* 1998), the data suggested it was not a primary factor in response to cellular stressors.

5.4 Concluding remarks

In this chapter, the transcript levels of total MR and the three variants in hypothermic anoxic neonatal rats were measured. The data shows that both hypothermia alone and anoxia&hypothermia caused a significant increase in the expression of total MR in the hippocampus of neonatal rat, which is contributed by the induction of MRβ variant solely. This corroborates the findings *in vitro* that MRβ is indeed the predominant variant responsible for the induction of total MR in hypothermic neuroprotection following cerebral ischaemic insult, being of substantial importance for the development of potential MR-based neuroprotectant in clinical treatment by targeting MRβ expression specifically.

Chapter 6

GENERAL DISCUSSION

It is well known that the essential function of MR is not only limited to regulating salt and water homeostasis in the kidney, but also linked to maintenance of neuronal viability in the brain after exposure to various stressful conditions such as cerebral ischaemia (Almeida *et al.* 2000; Macleod *et al.* 2003; Crochemore *et al.* 2005; Lai *et al.* 2007). This provides a novel strategy to develop the potential neuroprotectants based on enhancing MR signalling specifically in the neurons in conditions of ischaemic stroke or cardiac arrest.

However, how MR is regulated in such events is still unknown. Therefore, this thesis aims to answer this question by examining the expression of MR variants including promoter activity and mRNA abundance in three distinct systems -- cell line, primary cortical culture and *in vivo* animal models. The main findings are summarized as below:

(i) There is indeed a differential regulation of MR in response to specific cellular stressors.

(ii) MRβ is the primary sensor responsible for the induction of MR in hypothermic ischaemia in *in vitro* primary cortical culture and *in vivo* neonatal rat.

(iii) The induction of MRβ is initiated at promoter level.

The induction of MR responsive to specific cellular stressors is mediated by MRβ

Alternative RNA splicing is originally believed to expand the diversity of protein products from a single gene. Furthermore, it was later found that it can also generate multiple mRNA species differing in the 5'-untranslated regions but

encode the same protein, which is associated with a better control of gene expression in different tissues, organic developmental stages and other cellular activities (Graveley 2001). Subjected to such mechanism, rat MR gene gives rise to three mRNA variants with different transcription patterns in tissues and embryonic development (Patel *et al.* 1989; Kwak *et al.* 1993). Our group found that MR abundance was induced in rat primary cortical cultures by staurosporine treatment *in vitro* and hippocampus by hypothermic transient global ischaemia *in vivo* (Macleod *et al.* 2003), raising a novel mechanism that may also participate in the expression of endogenous MR.

The data demonstrate that the transcript levels of MR variants are differentially regulated in response to the cellular stressors and MRβ is verified to be the primary sensor specifically in hypothermic neuroprotection following ischaemic insults both *in vitro* and *in vivo*. This differential regulation of transcription in the context of brain injury is not unique to MR, for example, the neurotrophic factor BDNF gene gives rise to four mRNA variants each under the control of its own promoter, in a same manner as MR. Besides, the BDNF mRNA variants manifest distinct expression patterns in exposure to cerebral ischaemia *in vivo* (Tsukahara *et al.* 1998).

Currently, there is little information on the transcription regulation of MRβ variant in the rat. The expression of MRβ was barely detected in the kidney but share approximately same proportion with MRα in the adult rat hippocampus (Kwak *et al.* 1993). The abundance of MRβ was enriched in hippocampus during embryonic foetal development but declined rapidly in adult (Vazquez *et al.* 1998). The study in Chapter 4 also demonstrated that MRβ occupies over 50% of total MR transcripts in primary cortical culture, confirming it is a predominant variant in the young animals.

The mechanism of MRβ induction is not clear, but it might involve the cellular responses to brain injury that underlies the development (Chen *et al.* 2005). Given the role of MRβ in neuronal development, it is plausible to hypothesize that the induction of MRβ is responsive to such events. Surprisingly, hypothermia alone also up-regulated MRβ expression in neonatal rat *in vivo* which consequently leads to the induction of total MR. This might be due to either less sensitivity of neonatal rats in response to anoxia or inadequate severity of insult that is not enough to mount an MR-mediated survival response eventually.

More importantly, it appears that the induction MRβ expression is only confined to neuronal cells and responsive to neuronal injuries. This is supported by the findings that the increased MRβ promoter activity was found in neuronal-like differentiated PC12 cells and the transcript levels were up-regulated in primary cortical culture and brain hippocampus. It is a vital discovery for the future development of potential MR-based neuroprotectant, since the activation of MR in other tissues in particular heart and cerebral vessels could bring severe damage following cerebral ischaemia (Dorrance *et al.* 2006; Zhang *et al.* 2008). Further experiments testing MRβ expression in these tissues outside brain in response to the same cellular stressors are necessary to rule out the possible involvement of MRβ.

The induction of MRβ is initiated at the promoter level

Previous study has shown MR is nearly saturated at basal level of circulating corticosteroids in the brain (Reul *et al.* 1987). Therefore, the induction of MR signalling relies on an increase in the receptor availability instead of hormone level and this is believed to be initiated at gene transcription level. Assays of luciferase reporter gene under the direction of 300bp MRβ promoter fragment

demonstrated the promoter activity was significantly increased in response to OGD&hypothermia in neuronal-like differentiated PC12 cells *in vitro*, suggesting the induction of MRβ is initiated at the promoter level.

Probably due to lack of enhancers within such a short region, basal promoter activity of MRβ is lowest of three MR variants. To examine if the necessary element(s) responsible for the increased promoter activity in exposure to OGD&hypothermia is/are exclusively located in this 300bp region, two longer extension promoter constructs were generated and subjected to the same cellular stressor OGD&hypothermia using the same method as the experiments in Chapter 3 (Please refer to appendix 3). The basal promoter activity was significantly increased whereas the magnitude of induction by OGD&hypothermia was not changed between them, suggesting the potential element(s) is/and located within the first 300bp promoter region.

Elk-1 is a potential transcription factor responsible for MRβ induction

Our group has reported an association between increased MR mRNA and activation of the ERK pathway in primary neuronal cultures which were subjected to cell stress staurosporine (Macleod *et al.* 2003). Data using a variety of protein inhibitors or blockers demonstrated that inhibition of ERK pathway, can significantly attenuate OGD&hypothermia-induced MRβ promoter activity (Peng *et al.* in press), suggesting a potential involvement of ERK. Screening of 300bp MRβ promoter region reveals a putative transcription factor binding site for Elk-1, a downstream effector in ERK pathway.

Elk-1, a member of ternary complex family of ETS-related transcription factor, is fundamentally important for cellular proliferation, differentiation, development and transformation (Sharrocks 2001; Oikawa *et al.* 2003). In central nervous system, Elk-1 gene is strongly expressed in the brain and restricted in neuronal

cells (Janknecht *et al.* 1994; Pingoud *et al.* 1994; Price *et al.* 1995). Stress-inducing multiple factors in MARK pathways like ERK, JNK and p38 are able to activate Elk-1 by phosphorylation (Janknecht *et al.* 1994; Cavigelli *et al.* 1995; Gille *et al.* 1995; Enslen *et al.* 1998; Ferrer *et al.* 2001). Particularly the phosphorylated Elk-1 was up-regulated in hypoxia model *in vitro* (Muller *et al.* 1997) and later was reported in animal models in both focal and cerebral ischaemia (Wang *et al.* 2004; Krupinski *et al.* 2005). Indirect evidence comes from the coincidence of hypothermic neuroprotection with the activation of ERK pathway (Hicks *et al.* 2000; D'Cruz *et al.* 2002), which possibly act through Elk-1. Taken together, it strongly suggested that activation of Elk-1 induces MRβ expression but further experiments using promoter deletion constructs, DNase footprinting and EMSA are necessary for the validation of this hypothesis.

MRα is not a sensor although the promoter is increased

During foetal development, MRα was consistently expressed at a lower level in comparison to MRβ in the hippocampus (Vazquez *et al.* 1998). However, in adult rat MRα is the predominant variant in kidney and shares a same proportion with MRβ at approximately 30% in the hippocampus (Kwak *et al.* 1993). The basal promoter activity is significantly higher than MRβ and MRγ in both undifferentiated and differentiated PC12 cells, suggesting it is a fairly active variant in terms of transcriptional activity. Both staurosporine and OGD&hypothermia caused an increase in MRα promoter activity in PC12 cells, the transcript levels were not altered in the model systems either *in vitro* or *in vivo*. This is probably due to the intrinsic properties of MRα variant such as mRNA stability and degradation rate, or the RNA processing like post-transcriptional modification.

Up-regulation of MRβ -- a role in aging animals?

In neonatal rats, the up-regulation of MRβ in exposure to OGD&hypothermia was found in hippocampus over the five subfields, accounting for the induction of total MR. Given the notion that there is a highly homology in pathophysiology between cerebral ischaemia and cerebral anoxia in animal models (Vannucci 1990; Johnston *et al.* 2001), this is of clinical relevance because the condition here mimicks the hypothermic treatment for the ischaemic patients. Therefore, specific stimulation of MRβ expression can act as an effective endogenous factor which initiates the survival signalling in a same manner as hypothermia to protect the neurons from death.

However, epidemiological study on human cerebral ischaemia demonstrated that the vast majority of ischaemic episodes occurring in those aged ≥65 years (Sudlow *et al.* 1997; Wolfe 2000). Substantial loss of neurons and altered cellular activities are exclusively present in the aging brain (Crain *et al.* 1988). Besides, pre-clinical research also reported that aged animals are more vulnerable to ischaemic insult and may lack the sensitivity to the treatment, probably due to reduced energy utilization, accumulation of oxidation and increased GC levels (Xu *et al.* 2007). Besides, the expression of hippocampal MR in aged animals was significantly reduced in comparison to adult (Sapolsky *et al.* 1983; Reul *et al.* 1988), which may account for loss of neurons as well. Taken together, this might compromise the potential changes of MRβ expression due to lack of efficient endogenous survival mechanisms that can switch on MRβ. An exploration on the transcript levels of MR variants in aged animals in exposure to hypothermic cerebral ischaemia would certainly be helpful towards the thorough understanding of transcription regulation of MR.

In comparison to previous neuroprotectants examined in pre-clinical research, MR

has several unique advantages. (i) As a transcriptional factor, MR is known to regulate a number of genes downstream including up-regulating anti-apoptotic genes Bcl-2 and Bcl-xl (Almeida *et al.* 2000; McCullers *et al.* 2001), neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) (Hansson *et al.* 2000) and down-regulating NMDA receptor expression (Nair *et al.* 1998). There can effectively trigger a number of efferent effects to promote neuronal survival. (ii) MR is an endogenous survival factor which may be involved in hypothermic neuroprotection. Hypothermia is well known for its neuroprotective role and has been widely used in clinical treatment for stroke patients. However, there are some limitations to the utilization of such intervention including side-effects and optimizing conditions for every patient (General introduction 1.2.5.2). Since hypothermia induced the expression of MR in transient global ischemia *in vivo* (Macleod *et al.* 2003), the neuroprotection of MR may share the same mechanism with hypothermia. Thus enhancing MR signalling can be a more potent target to exert the hypothermic protective effects. (iii) Human or rat MR gene gives rise to multiple mRNA transcripts which have distinct transcriptional expression pattern in different tissues or developmental stages (Kwak *et al.* 1993; Zennaro *et al.* 1995; Zennaro *et al.* 1997; Vazquez *et al.* 1998). In addition, this thesis demonstrated that these transcripts are also differentially regulated in an injury-specific manner. Hence, MR gene expression can be up-regulated specifically in neurons in response to cerebral ischemic insult, avoiding the potential deleterious effects caused by MR activation in other tissues such as vascular system.

In this thesis, the transcription regulation of MR variants in exposure to different types of cellular stress was investigated. The promoter activity, mRNA levels were measured for each of MR variants in *in vitro* and *in vivo* ischaemic models. MRβ was identified as a primary effector involved in hypothermic neuroprotection

during cerebral ischaemic-hypoxic damage. Therefore, up-regulation of MRβ variant specifically in the neurons can serve as a novel and suitable target for future development of neuroprotective drug to enhance the MR-mediated survival signalling.

Appendix 1: Generation of neonatal rat anoxia

Neonatal brains exposed to hypothermic anoxia were generated through a collaboration with Dr. Justyna Rogalska, N. Copernicus University, Torun Poland (Caputa *et al.* 2001).

Briefly, 2-day old Wistar rat pups weighing 7-8g were placed in temperature controlled plethysmographic chambers either at 33°C (normal body temperature of neonatal rats) or 31°C (hypothermia). Rectal temperature was recorded and as soon as the desired body temperature was reached, anoxia was elicited to the animals by flushing 100% nitrogen into the chambers. Respiratory efforts (gasping movements) were continuously recorded throughout the experiments using the barometric method. Animals were maintained were maintained under anoxic conditions for 25 minutes. After anoxia, animals were exposed to atmospheric air at unchanged temperature for 120 minutes then sacrificed. Brains were dissected and immediately frozen on dry ice. Control rats were placed in chambers and exposed to atmospheric air for the same period of time (145 minutes) under the respective temperature conditions.

Appendix 2: Measurement of cell death by LDH assay

The percentage of cell damage was calculated according to the following equation:

$$\text{Death percentage (\%)} = \frac{\text{Prelysis[LDH]}}{\text{Total [LDH]}} \times 100 \quad (1)$$

Total [LDH] = New released [LDH] + prelysis[LDH] 1

Because the addition of 50ul of medium will dilute the current LDH before cell lysis occurs by a factor of 150/200, the postlysis [LDH] can not be recognized simply as the Total[LDH], but

Measured [LDH]=Newly released [LDH] + 150/200 prelysis [LDH] 2

Therefore,

Newly released LDH= Measured[LDH] – 150/200 prelysis [LDH] 3

If we substitute the newly released[LDH] in equation 1 with the one in 3, then,

Total [LDH]= Measured[LDH] – 150/200 prelysis[LDH] + prelysis[LDH]

=Measured[LDH] + 1/4 prelysis[LDH]

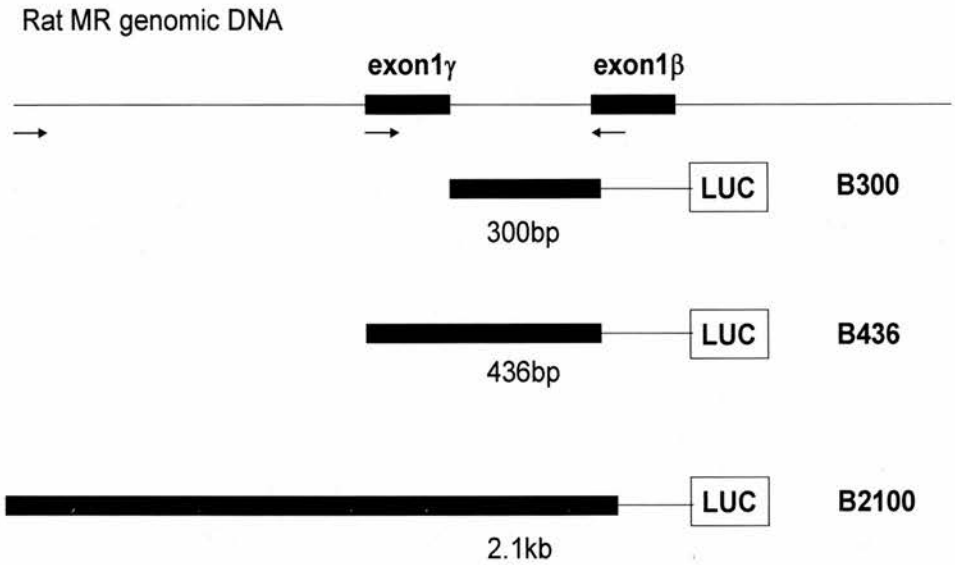
Finally, we got

$$\text{Death percentage (\%)} = \frac{\text{Prelysis[LDH]}}{\text{Meaured[LDH]} + 1/4\ \text{prelysis[LDH]}} \times 100$$

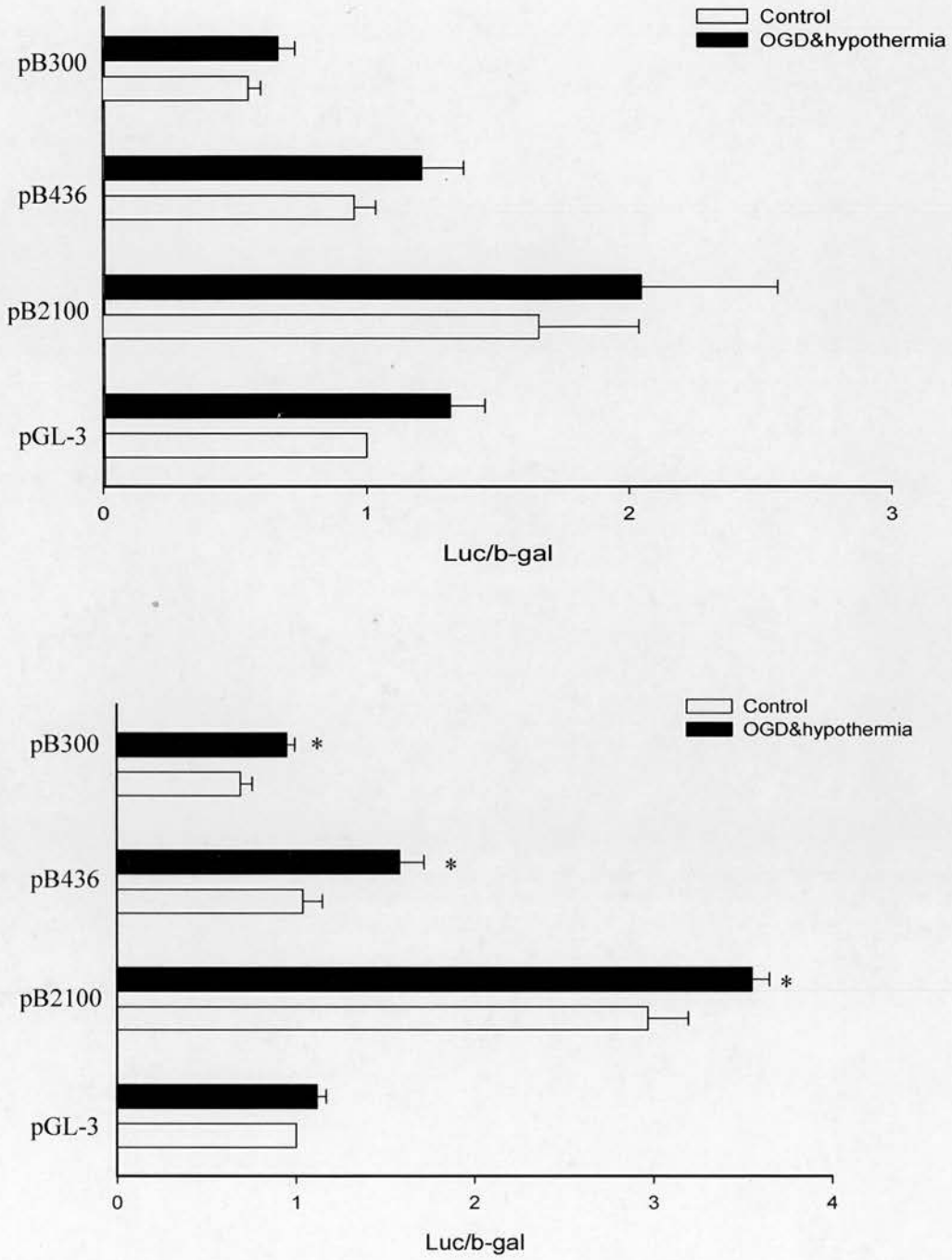
Appendix 3: Promoter activity of pMRβ extension constructs in response to OGD&hypothermia in undifferentiated and differentiated PC12 cells

To validate the increased promoter activity of MPβ after exposure to OGD&hypothermia was mediated by its unique 300bp promoter region, two longer promoter fragments, pB2100 with a length of 2.1kb and pB436 with 436bp, were individually amplified from rat genomic DNA and sub-cloned into pGL-3 vector. The method was same as stated using transient transfection into undifferentiated and differentiated PC12 cells in response to OGD&hypothermia.

In both undifferentiated and differentiated PC12 cells, the longest construct pB2100 showed the highest basal promoter activity, while pB436 activity was lower than pB2100 but higher than the original pB300. OGD&hypothermia did not increase the promoter activity of pB2100, pB436 or pB300 in undifferentiated PC12 cells. However, in differentiated PC12 cells, the promoter activity was significantly increased from 2.96 to 3.55 for pB2100 (120%± 3.3%, $p<0.05$), 1.04 to 1.58 fold for pB436 (150%± 13%, $p<0.05$) and 0.69 to 0.96 fold for pB300 (138%±6.6% $p<0.05$).

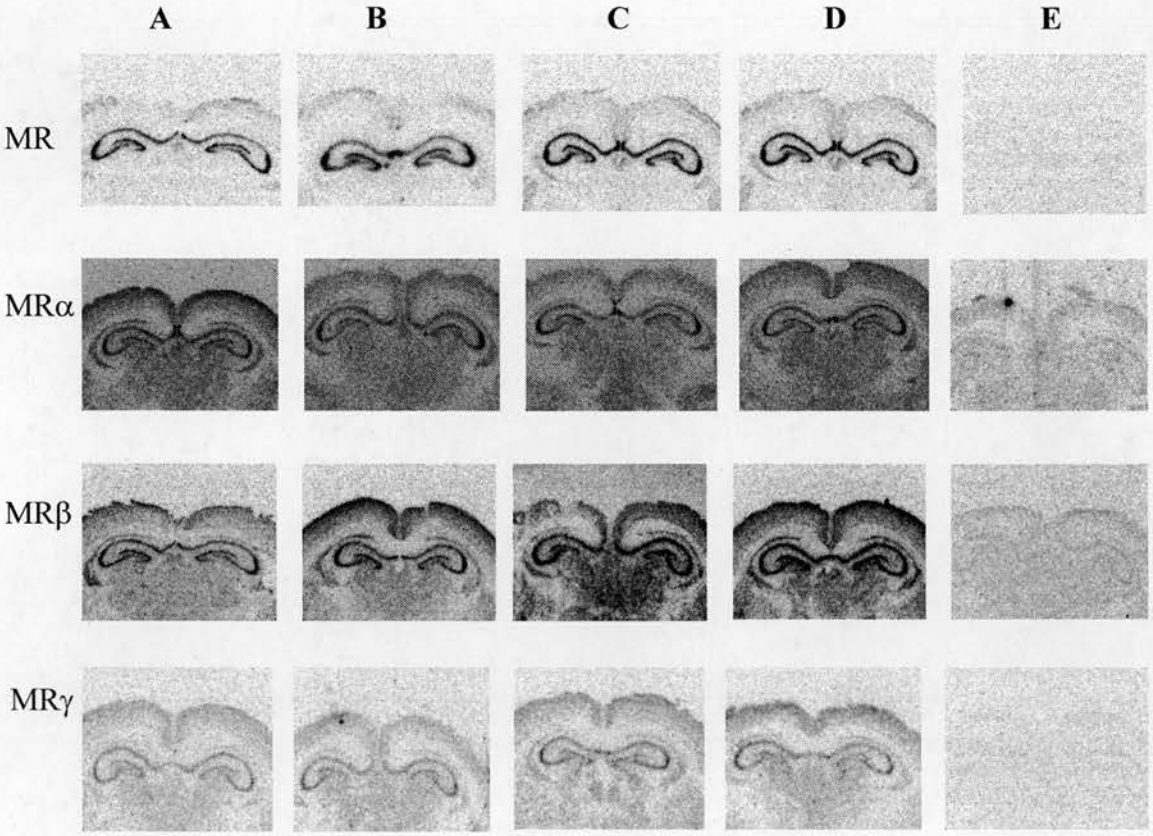


Schematic illustration of MRβ promoter extension constructs.



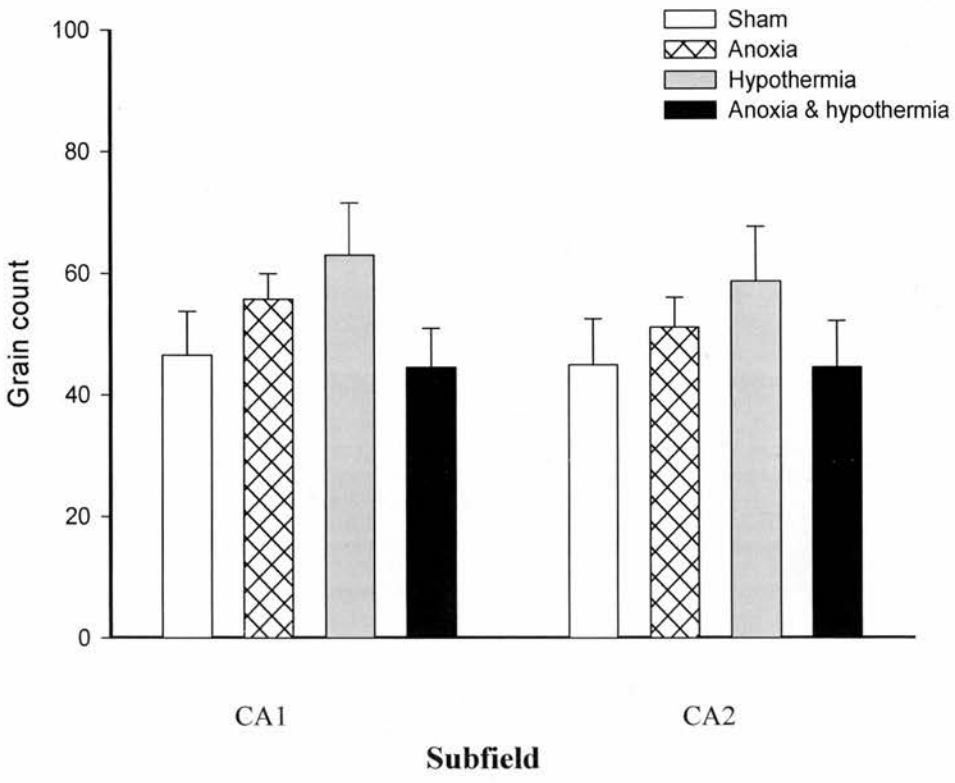
Promoter activity of MRβ extension constructs in response to OGD&hypothermia in undifferentiated (A) and differentiated (B) PC12 cells. None of MRβ constructs were significantly altered by OGD&hypothermia in undifferentiated cells, whereas they were significantly increased in differentiated cells. *$p<0.05$, Mean $\pm$ S.E.M, n=5 independent experiments each performed in triplicate.

Appendix 4: Autoradiographic pictures of *in situ* hybridization



Expression of MR and its variants in hippocampus in hypothermic anoxic neonatal rat. Column A: sham; Column B: anoxia; Column C: hypothermia; Column D: anoxia&hypothermia; Column E: sense

Appendix 5: Effect of hypothermic anoxia on GR expression in neonatal rats



Effect of hypothermic anoxia on GR expression in neornatl rat. There was no significant difference in GR expression between treatment group and sham group in any of the hippocampal subfield. Data represent mean ± S.E.M, n=6 animals per group with each performed in triplicate brain sections.

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